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Design of Unnatural Isocarbostyril and 2,4-Difluorophenyl Pyrimidine Nucleoside Mimics, and Nitric Oxide Donor Nitrate Esters of Pyrimidine Nucleosides for Evaluation as Anticancer and Antiviral Agents

by

Ebrahim Naimi

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Dedicated to:

My mother who taught me how to love and my father who taught me how to fight for my goals.

My wife, Mehrnoush, my children, Samin and Armin,

and

My brothers, Mostafa, Morteza and Mojtaba,

For their constant encouragement and support during my Ph.D. program.

ABSTRACT

A group of unnatural 1-(2-deoxy-β-D-ribofuranosyl)isocarbostyrils having a variety of C-7 substituents [H, 4,7-$(NO_2)_2$, I, CF_3, CN, (*E*)-CH=CH-I, -C≡CH, -C≡C-I, -C≡C-Br, -C≡C-Me], designed as nucleoside mimics, were synthesized for evaluation as anticancer and antiviral agents. This class of compounds exhibited weak cytotoxicity in a MTT assay (CC_{50} = 10^{-3} to 10^{-5} M range), with the 4,7-dinitro derivative (**9**) being the most cytotoxic, relative to thymidine (CC_{50} = 10^{-3} to 10^{-5} M range), against a variety of cancer cell lines. The 4,7-dinitro (**9**), 7-I (**11**) and 7-C≡CH (**20**) compounds exhibited similar cytotoxicity against non-transfected (KBALB, 143B), and HSV-1 TK^+ gene transfected (KBALB-STK, 143B-LTK) cancer cell lines possessing the herpes simplex virus type 1 (HSV-1) thymidine kinase gene (TK^+). This observation indicates that these compounds probably are not substrates or weak substrates for HSV type-1 TK, or may be good substrates but weak binders and are therefore unlikely to be useful in gene therapy based on the HSV gene therapy paradigm.

In a second study, a group of 3'-*O*-nitro-2'-deoxyuridines (**35**), 3'-*O*-nitro-2'-deoxycytidines (**38**) and 5'-*O*-nitro-2'-deoxyuridines (**40**) possessing a variety of substituents (H, Me, F, I) at the C-5 position were synthesized for evaluation as anticancer/antiviral agents that have the ability to concomitantly release concentration dependent cytotoxic nitric oxide (NO). Compounds **35**, **38** and **40** generally released a greater percent of NO than the reference drug isosorbide dinitrate, that contains two nitrooxy substituents, upon incubation in the presence of either 18 mM L-cysteine (0.0-5.4% range at 1 hour, and 3.8-59.9% range at 16 hours relative to 3.5% at 1 hour and 24.0% at 16 hours for isosorbide dinitrate), or serum (2.9-47.1% range at 1 hour, and 4.4-

59.5% range at 16 hours relative to 1.9% at 1 hour and 2.6% at 16 hours for isosorbide dinitrate). Nitric oxide release in phosphate buffer alone was negligible (< 0.05% at 1 hour and < 1.0% at 16 hours). These 3'- and 5'-*O*-nitro-pyrimidine nucleosides (**35, 38, 40**) exhibited comparable cytotoxicity (CC_{50} = 10^{-3} to 10^{-6} M range) to 5-iodo-2'-deoxyuridine, but weak cytotoxicity relative to 5-fluoro-2'-deoxyuridine, against a variety of cancer cell lines. No differences in cytotoxicity against non-transfected (KBALB, 143B), and the corresponding transfected (KBALB-STK, 143B-LTK) cancer cell lines possessing the herpes simplex virus type 1 (HSV-1) thymidine kinase gene (TK^+) were observed, indicating that expression of the viral TK enzyme did not provide a gene therapeutic effect. Compounds **35, 38** and **40** lacked antiviral activity except for 5-iodo-3'-*O*-nitro-2'-deoxyuridine (**35d**), which showed some selectivity against HSV-1 (KOS), HSV-2 (G), and vaccinia virus (IC_{50} = 48 μg/mL).

In a third investigation, a group of 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-benzenes having a variety of C-5 substituents, designed as thymidine mimics, were synthesized for evaluation as antiviral and anticancer agents. The regiospecific addition of HOBr (generated from *N*-bromosuccinimide in aqueous dioxane) across the 5-vinyl substituent (**44**) afforded the corresponding 5-[-CH(OH)CH_2Br] product (**45**), whereas reaction of **44** with iodine in the presence of iodic acid (HOI) yielded the 5-[CH(OH)CH_2I] product (**46**). The related 5-[-CH(OH)CHX_2 (X = Br, I)] analogs (**51-52**) were similarly prepared from the (*E*)-5-(2-halovinyl) precursors (**49-50**). Treatment of the 5-[-CH(OH)CH_2X (X = Br, I)] compounds (**45, 46**) with NaOH in aqueous dioxane afforded the 5-oxiranyl product (**48**). The 5-[-CH(OMe)CH_2I] compound (**47**) was prepared by reaction of the 5-vinyl compound (**44**) with ICl in MeOH (MeOI). This

group of compounds (**45-48, 51-52**) showed similar (marginal) activity against varicella-zoster virus thymidine kinase positive (VZV/TK$^+$) and thymidine kinase deficient (VZV/TK$^-$) infected cells. Thus, the viral TK enzyme did not provide a gene therapeutic effect. This group of compounds that were evaluated using a wide variety of antiviral assay systems [herpes simplex virus (HSV-1, HSV-2), varicella-zoster virus (VZV), vaccinia virus, vesicular stomatitis, cytomegalovirus (CMV) and human immunodeficiency virus (HIV-1, HIV-2)], showed that these unnatural *C*-aryl nucleoside mimics are inactive antiviral agents. Their failure to exhibit antiviral/anticancer activity could be due to the fact that they are not phosphorylated to the 5'-monophosphate, or that incorporation of the active 5'-triphosphate into DNA does not produce a cytotoxic effect, and/or that these *C*-aryl nucleoside mimics do not act as inhibitors of thymidylate synthase, which may be required to produce a cytotoxic effect.

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List of Symbols, Nomenclature, or Abbreviations

A	Adenine, Adenosine
ACV	Acyclovir
AdoHcy	*S*-Adenosyl-L-homocysteine
ADP	Adenosine 5'-diphosphate
AG	Aminoguanidine
AICAR	5'-Phosphoribosyl-5-aminoimidazole-4-carboxamide
AMP	Adenosine 5'-monophosphate
Anal	Analysis
APRTase	Adenine phosphoryl transpherase
Ara-A	1-(β-D-arabinofuranosyl)adenine
Ara-C	1-(β-D-arabinofuranosyl)cytosine
Ara-G	9-(β-D-arabinofuranosyl)guanine
ATP	Adenosine 5'-triphosphate
AZT	3'-Azido-2',3'-dideoxythymidine
BBB	Blood-brain-barrier
BH_4	Tetrahydrobiopterin
BHCG	(R)-9-[2,3-Bis(hydroxymethyl)cyclobutyl]guanine
BHV	Bovine herpesvirus
br.	Broad
BTDU	5-(5-Bromothien-2-yl)-2'-deoxyuridine
BVaraU	(*E*)-5-(2-Bromovinyl)arabinouridine

BVDU	(*E*)-5-(2-Bromovinyl)-2'-deoxyuridine
°C	Degrees celsius
C	Cytosine, Cytidine
cm	Centimeter
^{13}C NMR	Carbon nuclear magnetic resonance spectroscopy
calcd	Calculated
C-BVDU	Carbocyclic BVDU
CC_{50}	Median cytotoxic concentration
$CCID_{50}$	Median cell culture inhibitory dose
C-Cyd	Cyclopentylcytosine
CD	Cytosine deaminase
CdA	2-Chloro-2'-deoxyadenosine
CDP	Cytidine 5'-diphosphate
CEDU	5-(2-Chloroethyl)-2'-deoxyuridine
cGMP	Cyclic guanosine monophosphate
CK	Cytidine kinase
CMP	Cytidine 5'-monophosphate
CMV	Cytomegalovirus
CTP	Cytidine 5'-triphosphate
CVDU	5-(2-Chlorovinyl)-2'-deoxyuridine
cycloSal	Cyclosaligenyl
Cys	Cysteine
Cyt-P450	Cytochrome P450

d	Doublet
D_4T	2',3'-Didehydro-3'-deoxythymidine
dA	2'-Deoxyadenosine
dADP	2'-Deoxyadenosine-5'-diphosphate
dAMP	2'-Deoxyadenosine-5'-monophosphate
dATP	2'-Deoxyadenosine-5'-triphosphate
dC	2'-Deoxycytidine
dCDP	2'-Deoxycytidine-5'-diphosphate
dCK	2'-Deoxycytidine kinase
dCMP	2'-Deoxycytidine-5'-monophosphate
dCTP	2'-Deoxycytidine-5'-triphosphate
ddC (DDC)	2',3'-Dideoxycytidine
DDI	2',3'-Dideoxyinosine
ddN (DDN)	2',3'-Dideoxynucleoside
DDNTP	2',3'-Dideoxynucleoside-5'-triphosphate
ddU	2',3'-Dideoxyuridine
dFdC	2',2'-Difluoro-2'-deoxycytidine
dFdG	2',2'-Difluoro-2'-deoxyguanosine
dG	2'-Deoxyguanosine
dGDP	2'-Deoxyguanosine-5'-diphosphate
dGK	2'-Deoxyguanosine kinase
dGMP	2'-Deoxyguanosine-5'-monophosphate
dGTP	2'-Deoxyguanosine-5'-triphosphate

DHFR	Dihydrofolate reductase
DIAD	Diisopropyl azodicarboxylate
DMAP	4-Dimethylaminopyridine
DMEM	Dulbeccos modification of eagles medium
DMF	*N,N*-Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleoside-5'-triphosphate
DP	Diphosphate
dTDP	Deoxythymidine 5'-diphosphate
dTMP	Deoxythymidine 5'-monophosphate
dTTP	Deoxythymidine 5'-triphosphate
dU	2'-Deoxyuridine
dUDP	2'-Deoxyuridine-5'-diphosphate
dUMP	2'-Deoxyuridine-5'-monophosphate
dUTP	2'-Deoxyuridine-5'-triphosphate
EC_{50}	Median effective concentration
E. coli	Escherichia coli
e.g.	For example
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EDU	5-Ethyl-2'-deoxyuridine
EHV	Equine herpesvirus

ELISA	Enzyme-linked immunosorbent assay
*e*NOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
eq	Equivalent
EtOAc	Ethyl acetate
FAD	Flavin adenine dinucleotide
F-AraA	2-Fluoro-(9-β-D-arabinofuranosyl)adenine
FBS	Fetal bovine serum
FdUMP	5-Fluoro-2'-deoxyuridine 5'-monophosphate
FIAU	1-(2-Deoxy-2-fluoro-β-D-arabinosyl)-5-iodouracil
FMN	Flavin mononucleotide
5-FU	5-Fluorouracil
FUdR	5-Fluoro-2'-deoxyuridine
G_1	Gap 1
G	Guanine, Guanosine
g	Gram(s)
GCV	Ganciclovir
GDN	Glyceryl dinitrate
GDP	Guanosine 5'-diphosphate
gem	Geminal
GMN	Glyceryl mononitrate
GMP	Guanosine 5'-monophosphate
GSH	Glutathione

GSNO	*S*-Nitrosoglutathione
GST	Glutathione-*S*-transferase
GTN	Glyceryl trinitrate
GTP	Guanosine 5'-triphosphate
h	Hour(s)
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
HBV	Hepatitis B virus
HEL	Human embryonic lung
HEPES	*N*-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HEPT	1-(2-Hydroxyethoxymethyl)-6-phenylthiothymidine
HGPRTase	Hypoxanthine-guanine phosphoryl transferase
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HOAc	Acetic acid
HPMPC	(S)-1-[3-Hydroxy-2-(phosphonyl-methoxy)propyl]cytosine
HPV	Human papillumavirus
HSV	Herpes simplex virus
HVP	Herpesvirus platyrrhinae
Hz	Hertz
IC_{50}	Median inhibitory concentration
ID_{50}	Median inhibitory dose
i.e.	That is

IFN	Interferon
IL	Interleukin
IMP	Inosine monophosphate
IMPDH	Inosine monophosphate dehydrogenase
*i*NOS	Inducible nitric oxide synthase
IR	Infra-red
ISDN	Isosorbide dinitrate
ISMO	Isosorbide 5-mononitrate
IUdR	5-Iodo-2'-deoxyuridine
IUPAC	International union of pure and applied chemistry
IVDU	5-(2-Iodovinyl)-2'-deoxyuridine
IVFRU	(*E*)-5-(2-Iodovinyl)-2'-fluoro-2'-deoxyuridine
KF	Klenow fragment
K_m	Michaelis constant
L	Liter(s)
L-NAME	N^{ω}-Nitro-L-arginine methylester
L-NMMA	N^{ω}-Monomethyl-L-arginine
LPS	Lipopolysaccharide
M	Mitosis
M	Molar concentration
m	Multiplet
mg	Milligram(s)
mg/kg	Milligram per kilogram

min	Minute(s)
mL	Milliliter(s)
mM	Millimolar concentration
mmol	Millimole(s)
mol	Mole(s)
MP	Monophosphate
mp	Melting point
mRNA	Messenger RNA
MS	Mass spectrometry
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide
MTX	Methotrexate
μ	Micro
μg	Microgram(s)
μL	Microliter(s)
μmol	Micromole(s)
N	Normal
NA	Nucleoside analog
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS	*N*-Bromosuccinimide
NDP	Nucleoside 5'-diphosphate

NDPK	Nucleoside diphosphate kinase
NED	*N*-(1-Naphthyl)ethylenediamine dihydrochloride
ng	Nanogram(s)
NIS	*N*-Iodosuccinimide
nm	Nanometer
NMPK	Nucleoside monophosphate kinase
NMR	Nuclear magnetic resonance spectroscopy
*n*NOS	Neuronal nitric oxide synthase
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NO	Nitric oxide
NOE	Nuclear Overhauser enhancement
NOS	Nitric oxide synthase
NTP	Nucleoside 5'-triphosphate
NUC	Nucleoside 5'-residue
OMP	Orotidine 5'-monophosphate
$P(Ph)_3$	Triphenylphosphine
PAU	5-(Prop-1-ynyl)arabinouridine
PBS	Phosphate-buffered saline
PCV	Penciclovir
PETN	Pentaerythrityl tetranitrate
PFU	Plaque forming units
pH	Log hydrogen ion concentration
PM3	A reparameterization of Austin model 1 (a semi-

	empirical method for molecular calculations)
PNP	Purine nucleoside phosphorylase
PPA	Phenyl phosphoralaninates
ppm	Parts per million (NMR)
PPRTase	Pyrimidine phosphoryl transferase
PRPP	5-Phosphoribosyl-pyrophosphate
PRTase	Phosphoryl transferase
q	Quartet
RNA	Ribonucleic acid
RNase	Ribonuclease
RR	Ribonucleotide reductase
RT	Reverse transcriptase
S	Synthesis
s	Singlet
SAH	*S*-Adenosyl-L-homocysteine
SARCs	Structure-activity relationship correlations
SATE	*S*-Acylthioethylphosphate
S-BVDU	4'-Thio-BVDU
SD	Standard deviation
SEM	Standard error of the mean
SHV	Swine herpesvirus
S_N2	Nucleophilic substitution bimolecular
SOD	Superoxide dismutase

SVV	Simian varicella virus
T	Thymine, Thymidine
t	Triplet
TASO-T	2',5'-Bis-O-(*tert*-butyldimethylsilyl)-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide)thymidine
T-cell	Thymus derived cell
TDP	Thymidine 5'-diphosphate
TFT	5-Trifluoromethyl-2'-deoxyuridine
THF	Tetrahydrofolate
THF	Tetrahydrofuran
CH_2THF	5,10-Methylene tetrahydrofolate
TK (tk)	Thymidine kinase
TLC	Thin layer chromatography
TMP	Thymidine 5'-monophosphate
TMS	Trimethyl silyl
TNF	Tumor necrosis factor
TP	Triphosphate
TS	Thymidylate synthase
TTP	Thymidine 5'-triphosphate
U	Uracil, Uridine
UDP	Uridine 5'-diphosphate
UMP	Uridine 5'-monophosphate
UTP	Uridine 5'-triphosphate

UV	Ultraviolet
v	Volume
vic	Vicinal
V_{max}	Maximum velocity
vs	Versus
VZV	Varicella Zoster virus
XGPRT	Xanthine-guanine phosphoribosyl transferase

1. INTRODUCTION

Cancer as a disease of cells

The term cancer refers to a heterogeneous group of diseases. Tumors arise from a single abnormal cell, which continues to divide indefinitely. The lack of growth controls, ability to invade local tissues, and ability to spread, or metastasize, are characteristics of cancer cells. These properties are not present in normal cells (Katzung, 2001).

Many factors have been implicated in the etiology of cancer (Gilman *et al.*, 1991). The most common of these factors are listed below:

1. *Viruses,* including Epstein-Barr virus (EBV), hepatitis B virus (HBV), and human papillomaviruses (HPV).
2. *Environmental and occupational exposures,* such as ionizing and ultraviolet radiation and exposure to chemicals, including vinyl chloride, benzene, and asbestos.
3. *Life-style factors,* such as high-fat, low-fiber diets, and tobacco and ethanol use.
4. *Medications,* including alkylating agents and immunosuppressants.
5. *Genetic factors,* including inherited mutations, cancer-causing genes (oncogenes), and tumor-suppressor genes.

Cell life cycle

A. Phases of the cell cycle of mitosis

Knowledge of the cell life cycle and cell cycle kinetics is essential to the understanding of the activity of chemotherapy agents in the treatment of cancer (Figure 1). A cell cycle is the period from the birth of a new cell to the time that the new cell

divides. DNA duplication is perhaps the most important doubling event in the entire cycle, because the genetic material of DNA must be copied exactly, and each daughter cell must receive a complete version. In a cell's nucleus, DNA replication occurs during only one specific part of the cell cycle, called the S (for synthesis) phase. Between division and S is a period called G_1 (meaning gap 1) or postmitotic gap, in which cells grow but do not make DNA. In G_1, many molecules such as enzymes, RNA and the proteins for the specialized functions of the cell are synthesized. Another period called G_2 or the premitotic or postsynthetic gap follows S, in which RNA and the enzymes topoisomerase I and II are produced. Finally in the fourth phase called M (mitosis), two DNA copies separate and two daughter cells are formed. Although each of these four main periods of the cell cycle (G_1, S, G_2, M) is unique, all in proper order are necessary for new cell production.

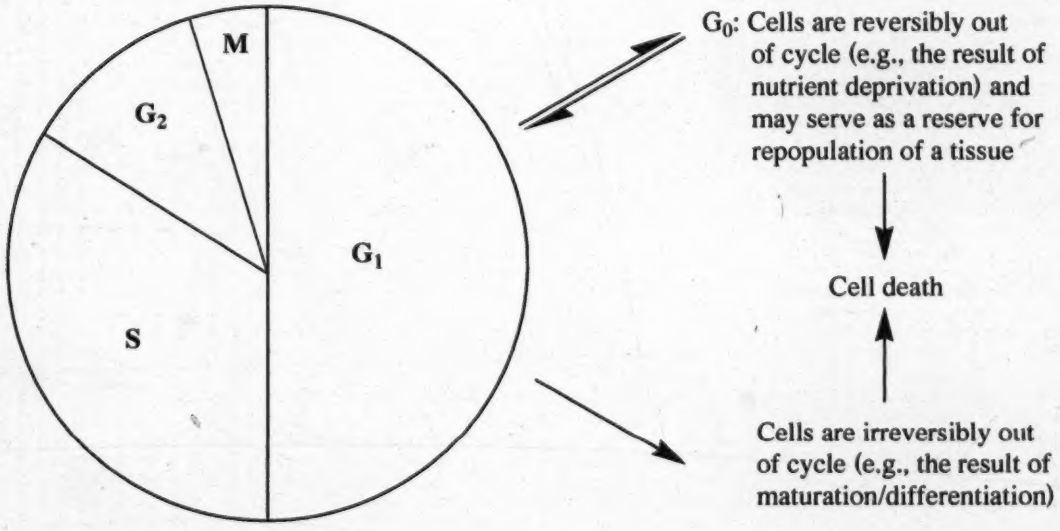

M
G2
G1
S
G0: Cells are reversibly out of cycle (e.g., the result of nutrient deprivation) and may serve as a reserve for repopulation of a tissue
Cell death
Cells are irreversibly out of cycle (e.g., the result of maturation/differentiation)

Figure 1. Diagrammatic representation of the cell growth cycle (Holleb, 1991).

Normally, cells are in either a growing state, in which they advance through the cell cycle, or a resting state, in which they continue to use energy, but do not synthesize DNA or divide. This resting state, called G_0, is distinct from the other steps in the cycle of an active cell. Normal cells usually pass into a resting state after M, but not after S or G_2. Resting cells most closely resemble G_1 cells in that they contain the same amount of DNA, which suggests that the G_1 state has a special, built-in mechanism that decides whether cells should proceed through the cycle or switch off into the G_0 state. Cells can return from the G_0 resting state back to G_1 if supplied with whatever they lack, such as hormones, or relieved of whatever might have been in oversupply (Foye *et al.*, 1995).

B. Cell growth kinetics

Several terms describe cell growth kinetics (Katzung, 2001):

1. *Cell growth fraction* is the proportion of cells in the tumor dividing or preparing to divide. As the tumor enlarges, the cell growth fraction decreases because a larger proportion of cells may not be able to obtain adequate nutrients and blood supply for replication.
2. *Cell cycle time* is the average time for a cell that has just completed mitosis to grow and again divide and again pass through mitosis. Cell cycle time is specific for each individual tumor.
3. *Tumor doubling time* is the time for the tumor to double in size. As the tumor gets larger, its doubling time gets longer because it contains a smaller proportion of actively dividing cells due to restrictions of space, nutrient availability, and blood supply.

4. The *Gompertzian growth curve* illustrates these cell growth concepts (Figure 2).

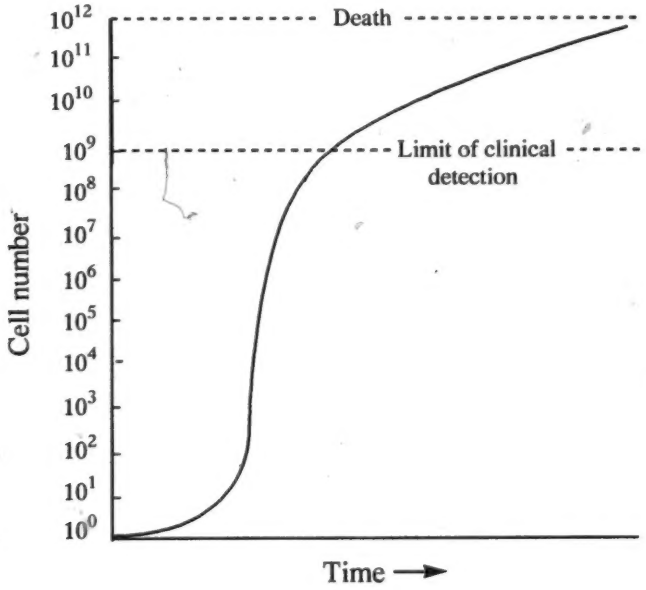


Figure 2. The Gompertzian growth curve (Holleb, 1991).

C. Tumor cell burden

Tumor cell burden is the number of tumor cells in the body. Because of the large number of cells required to produce clinical symptoms (approximately 10^9 cells), the tumor may be in the plateau phase of the growth curve by the time it is detected. Each cycle of cancer chemotherapy kills a certain percentage of the tumor cells. As tumor cells are killed, cells in G_0 may be recruited into G_1, resulting in tumor growth. Thus, repeated cycles of chemotherapy are required to achieve a complete response or remission. The percentage of cells killed is dependent on the chemotherapy dose (Holleb, 1991).

D. Chemotherapeutic agents

Chemotherapeutic agents may be classified according to their reliance on cell cycle kinetics for their cytotoxic effect. Combinations of chemotherapy agents that are active in different phases of the cell cycle may result in a greater cell kill. A cell cycle classification of some commonly used chemotherapeutic agents is listed below (Holleb, 1991):

1. *Phase-specific agents* are most active against cells that are in a specific phase of the cell cycle. For example:
    a. M phase: vinca alkaloids, taxanes
    b. G_1 phase: asparaginase, prednisone
    c. S phase: antimetabolites
    d. G_2 phase: bleomycin, etoposide
2. *Phase-nonspecific agents* are effective while cells are in the active cycle but do not require that the cell be in a particular phase. Examples include alkylating agents, antitumor antibiotics, and cisplatin.
3. *Cell cycle-nonspecific agents* are effective in all phases, including G_0. Examples include nitrosoureas and radiation.

Antimetabolites inhibit a metabolic pathway essential for the survival or reproduction of cancer cells through inhibition of folate, purine, pyrimidine, and pyrimidine nucleoside pathways required for DNA synthesis. No metabolic pathway unique for cancer cells has been found, yet antimetabolites can be used to kill tumor cells without killing the host, because of differences in cell growth fractions described previously (Katzung, 2001).

Chemistry of purines, pyrimidines, their nucleosides, and nucleotides

Heterocyclic compounds are ring (cyclic) compounds that contain both carbon and noncarbon (hetero) atoms. While heterocycles that contain sulfur or oxygen are important both biologically and therapeutically, the common hetero atom in biology is nitrogen. Purines and pyrimidines constitute a class of nitrogen-containing heterocycles of major biological importance. Their principal derivatives are nucleosides and nucleotides, both of which contain a cyclic sugar, usually a pentose, linked to a nitrogen hetero atom by a β-*N*-glycosidic bond. Nucleotides contain, in addition, one or more phosphoryl groups esterified to hydroxy groups of the sugar. The purine and pyrimidine moieties are essentially planar molecules that are numbered as shown in Figure 3 (Murray *et al.*, 1996).

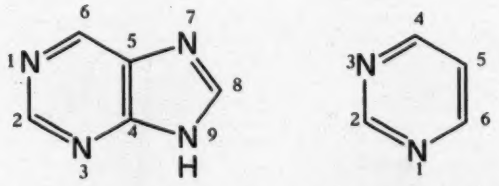


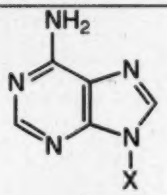
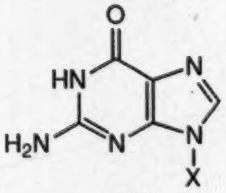
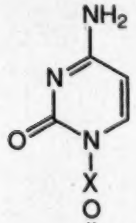
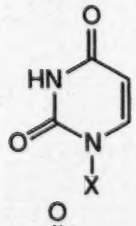
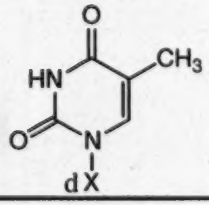
Figure 3. Purine and pyrimidine structures.

The structures, names, and abbreviations of common bases, nucleosides, and nucleotides are given in Table 1.

Nucleosides consist of a purine or pyrimidine base and a cyclic sugar, most often D-ribose or 2-deoxy-D-ribose, linked via a covalent, β-*N*-glycosidic bond to N-9 of purine or to N-1 of a pyrimidine. Numbering of the sugar atoms employs a prime to

distinguish sugar atoms from those of the heterocyclic base. Nucleotides are termed ribonucleotides or deoxyribonucleotides based on whether the sugar is ribose or 2-deoxyribose (Adams *et al.*, 1986).

Table 1. Names and abbreviations of nucleic acid bases, nucleosides, and nucleotides.

Base Formula	*Base (X = H)*	*Nucleoside (X = ribose or deoxyribose)*	*Nucleotide (X = ribose phosphate)*
	Adenine A	Adenosine A	Adenosine monophosphate AMP
	Guanine G	Guanosine G	Guanosine monophosphate GMP
	Cytosine C	Cytidine C	Cytidine monophosphate CMP
	Uracil U	Uridine U	Uridine monophosphate UMP
	Thymine T	Thymidine T	Thymidine monophosphate TMP

Tautomerism of purines and pyrimidines

Oxygenated purines and pyrimidines exist as keto and enol tautomeric structures with the keto tautomer being the major tautomer involved in hydrogen bonding between the bases in the nucleic acid RNA and DNA. The amine containing bases adenine and cytosine also exist as tautomeric structures with the amino form predominating as the tautomer involved in hydrogen bonding between the nucleic acid bases (Figure 4) (Voet *et al.*, 1999).

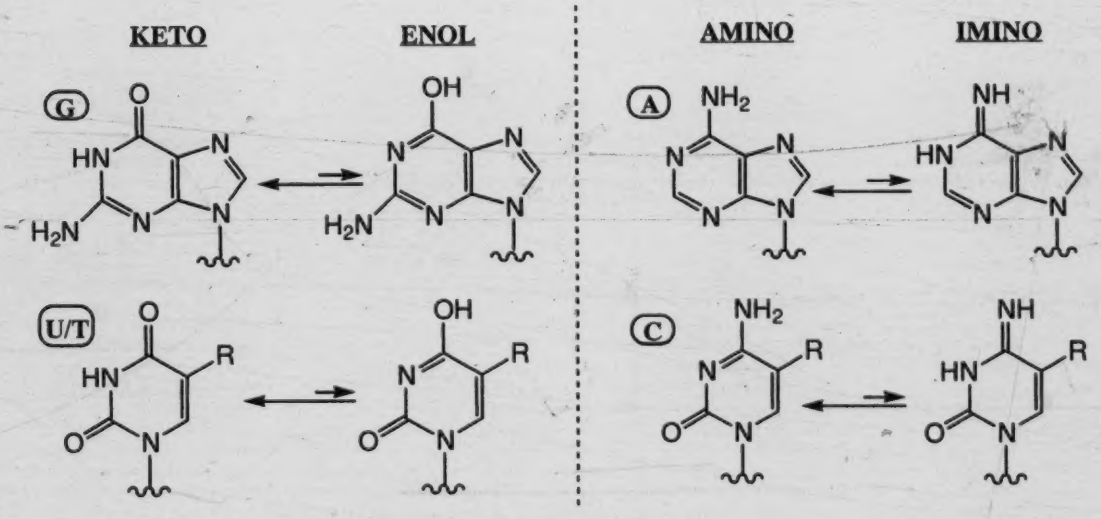


Figure 4. Tautomers of purines and pyrimidines

Nucleoside and nucleotide conformations

Steric hindrance by the heterocyclic base dictates that once formed, there is no freedom of rotation about the β-*N*-glycosidic bond that links sugars to purines or pyrimidines (Murray *et al.*, 1996). Nucleosides and nucleotides exist as stable, non-interconvertible *syn* (S-type) and *anti* (N-type) conformers (Figure 5) that can only be

interconverted by rupture and re-formation of the glycosidic bond. While both conformers occur in nature, *anti* conformers predominate, and it is the *anti* conformers of nucleotides that participate in normal base pairing in double-stranded DNA (Murray *et al.*, 1996).

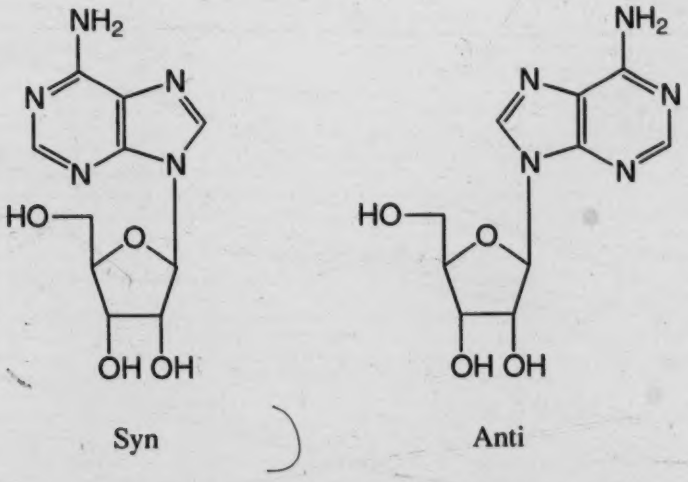


Figure 5. The *syn* and *anti* conformers of adenosine.

Nomenclature

Nucleosides are named with a mix of trivial and IUPAC nomenclature (McNaught, 1996) and very often abbreviated names are used. Both the trivial and systematic nomenclature for the nucleosides of RNA and DNA are shown in Figure 6. In IUPAC nomenclature, the sugar is described first with the position of attachment to the base indicated by the number in front of the sugar, followed by the name of the heterocyclic base.

Spectroscopy

There are many analytical techniques available for the structure elucidation of nucleosides such as infra-red (IR) and ultraviolet (UV) spectroscopy, and X-Ray

crystallography. However, the most useful techniques are Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy. NMR methods including ^{1}H, ^{13}C, and NOE (Nuclear Overhauser Enhancement) have been used mainly to provide a qualitative determination of the glycosidic bond/sugar conformation and general structure characterization.

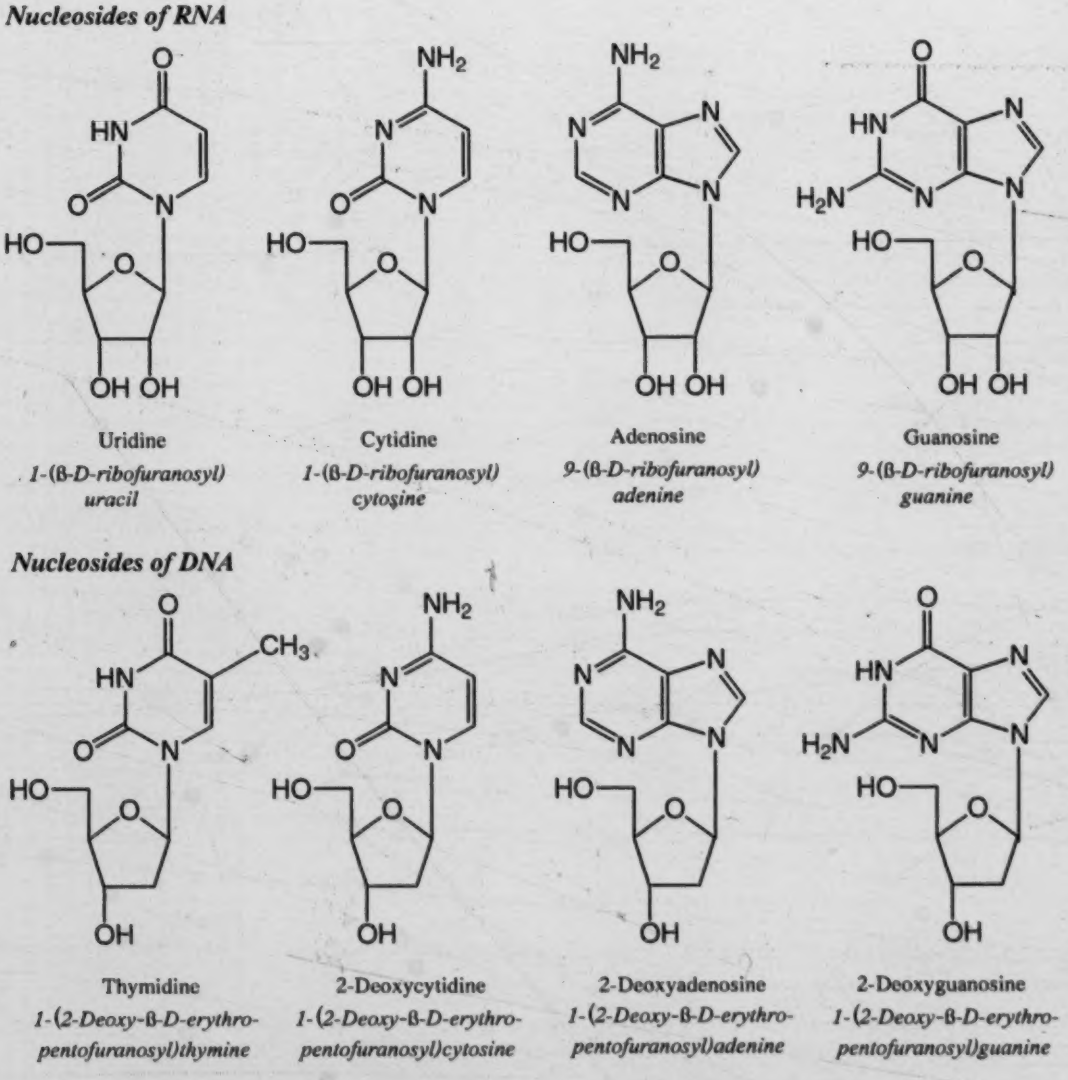


Figure 6. Nomenclature of the nucleosides of RNA and DNA.

^{1}H NMR Spectroscopy

Proton NMR spectra of the naturally occurring pyrimidine and purine nucleosides were determined in the 1960's and 1970's. Studies have provided information on sugar conformation (*syn/anti*, α/β-glycosidic configuration) and base character (tautomerism, H-bonding, base stacking) (Jardetzky & Roberts, 1981).

The H-1' in ribose occurs at a higher field than deoxyribose owing to increased electrostatic shielding of the ribose H-1' by the 2'-hydroxy group. The ribose H-1' appears as a doublet and the H-1' in deoxyribose appears as a triplet as a result of spin-spin interaction with one and two C-2' protons, respectively. H-4' and H-5' chemical shifts are reasonably constant in all the nucleosides, with H-3' well separated and downfield of H-4' and H-5'. Uridine and cytidine are exceptions with a smaller separation between H-3' and H-4'/H-5' owing to increased shielding. In the pyrimidine nucleosides H-6, being less shielded, occurs downfield of H-5.

Anomeric determination

NOE spectroscopy can be used to determine the anomeric configuration of nucleosides (Jardetzky & Roberts, 1981). In β-anomers, with N-type (C-2' endo) or S-type (C-3' endo) conformation, irradiation of H-1'often results in an NOE of the H-4' signal as both H-1' and H-4' are situated on the same face (α) of the sugar molecule. In α-anomers irradiation of H-1' often results in an NOE of the H-3' signal, with N-type conformers exhibiting a stronger enhancement than S-type conformers owing to the closer spatial proximity of H-1' and H-3' in N-type conformers.

^{13}C NMR Spectroscopy

The carbon-13 resonances of nucleosides can be divided into two distinct regions, those owing to the sugar moiety ($\leq$ 90 ppm) and those of the heterocyclic base ($\geq$ 90 ppm). The greatest difference in the ^{13}C spectra of ribose and 2'-deoxyribose nucleosides is the C-2' resonance signals. Exchanging the 2'-hydroxy group (~70 ppm) for a proton results in an upfield shift (~40 ppm), this also results in a β-substituent effect with the C-1' and C-3' resonances in deoxyribose which are shifted upfield 3-4 ppm relative to their positions in the ribose spectra. The characteristic signals of the pyrimidine base are the C5-C6 resonances. In all the pyrimidine nucleosides C-6 occurs downfield of C-5 as a result of the β-effect in alkenes. A downfield shift in C-5 of thymidine, consistent with a CH_3-substituted carbon, distinguishes thymidine from uridine.

Biological properties

Nucleosides and their derivatives are involved in a diverse range of biological processes such as energy metabolism, with adenosine 5'-triphosphate (ATP) being the principal form of chemical energy available to cells, as monomeric units of RNA and DNA and components of coenzymes such as nicotinamide adenine dinucleotide (NAD^+), and as physiological mediators, for example, adenosine 5'-diphosphate (ADP) being essential for platelet aggregation and guanosine 5'-triphosphate (GTP) being necessary for capping of messenger RNA (mRNA). The 5'-monophosphates (nucleotides) of the nucleosides in Figure 6 are the monomeric units of RNA (Figure 7, R = OH, R' = H) and DNA (Figure 7, R = H, R' = CH_3). The double stranded complex of these nucleic acids is held together by hydrogen bonds between the pyrimidine and purine bases, with three

hydrogen bonds between cytidine and guanine and two between adenine and either uracil or thymine, is known as Watson-Crick base pairing.

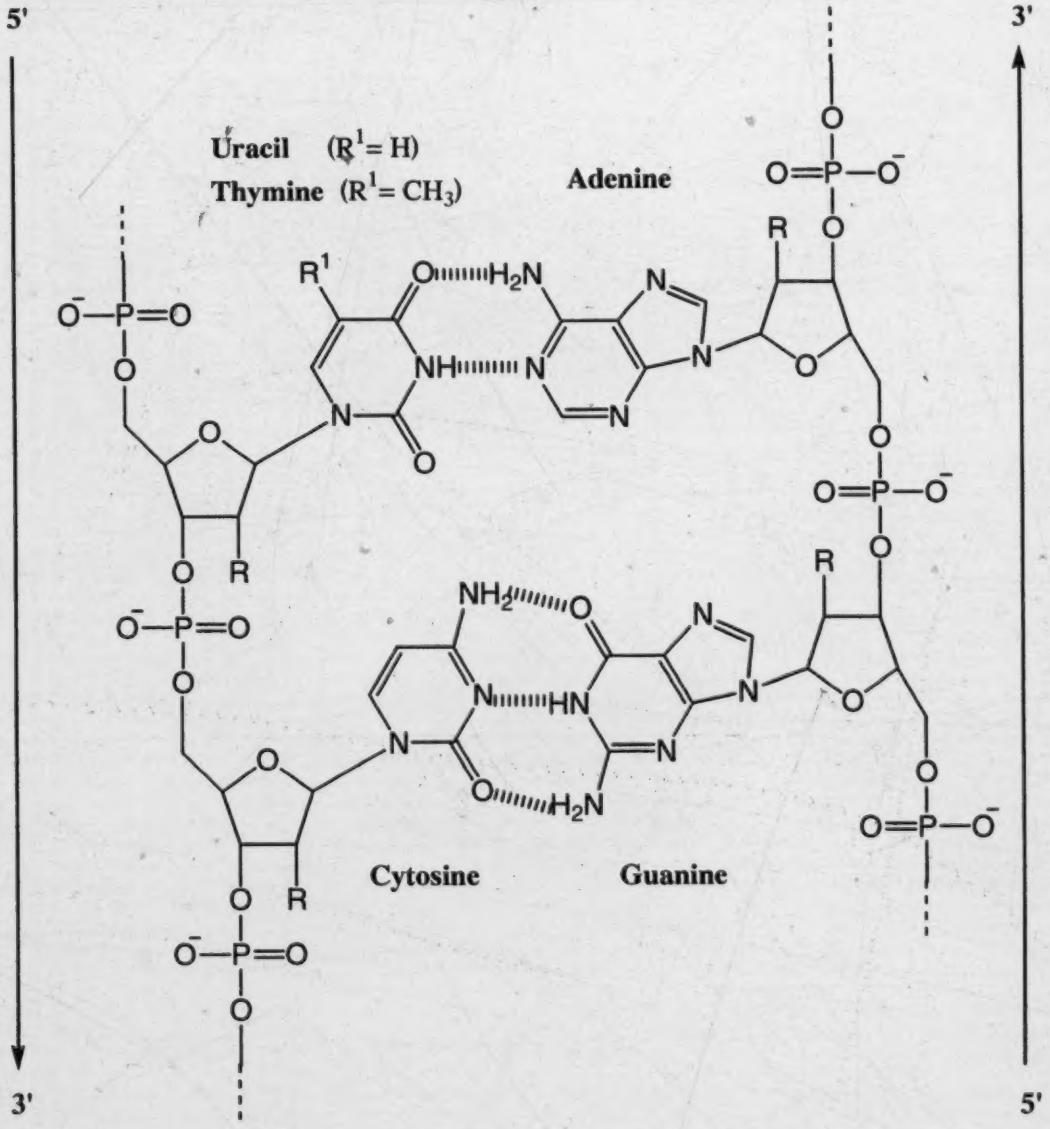


Figure 7. Structure of RNA and DNA double helix.

The nucleotides in the individual strands are linked by a phosphate backbone from a 3'-OH of one nucleotide to a 5'-OH of a second nucleotide, described as a 3'→5'

phosphodiester bridge. Additional nucleotides are added on at the 3'-OH position, therefore a free hydroxyl at the 3'-position is essential for chain extension.

Purine and pyrimidine nucleotide biosynthesis

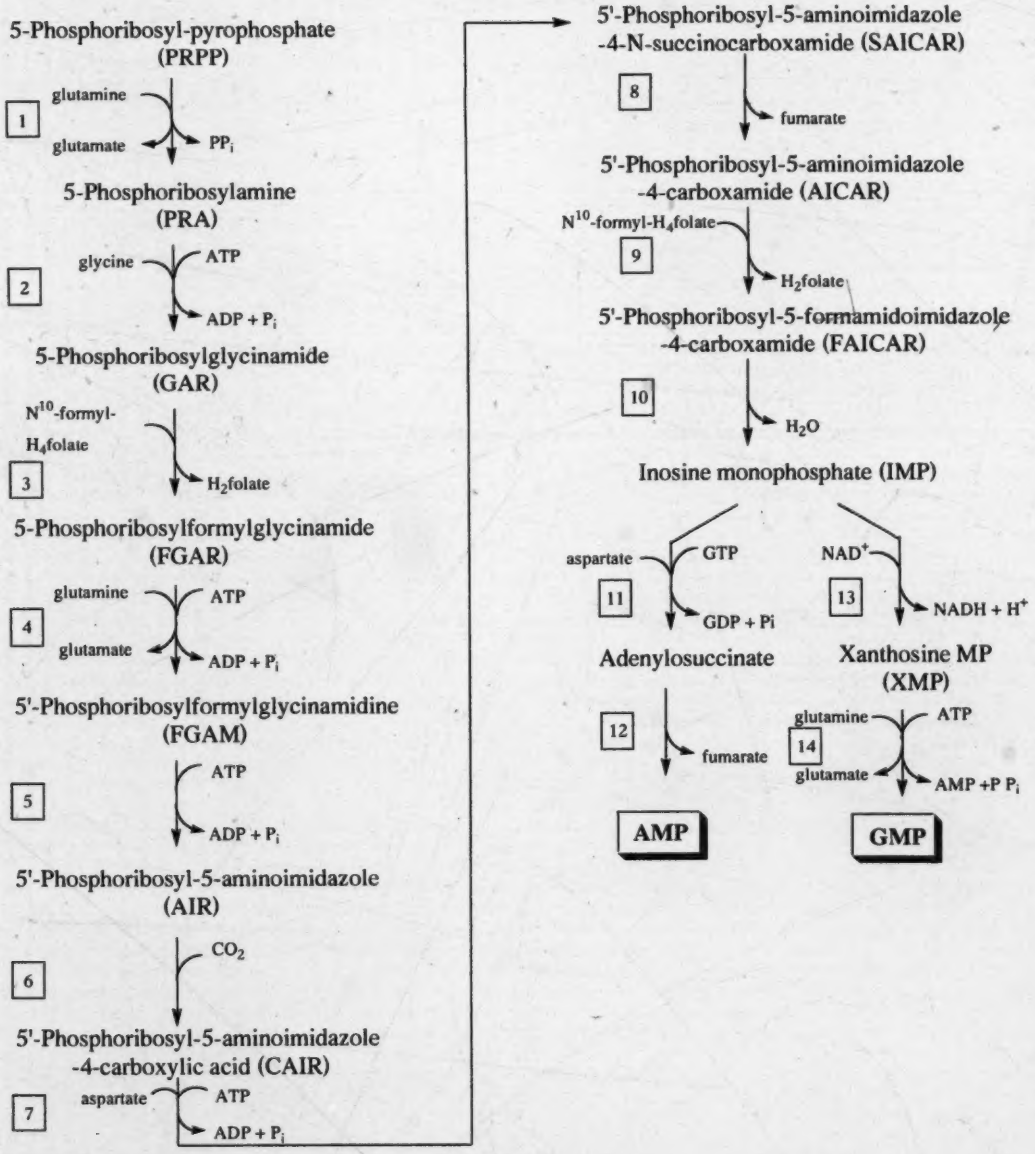
The 5'-nucleoside derivatives are the main purine and pyrimidine compounds found in cells, with ATP occurring in the highest concentration. Both purine and pyrimidine nucleotide synthesis is highly regulated, leading to fixed levels of the nucleotides, which is essential for the metabolic processes in which they are involved (Zalkin & Dixon, 1992; Cory, 1996).

***De novo* purine nucleotide synthesis**

The *de novo* pathway starts from 5-phosphoribosyl-pyrophosphate (PRPP) and involves a series of reactions leading to the formation of inosine monophosphate (IMP) *via* the imidazole nucleotide 5'-phosphoribosyl-5-aminoimidazole-4-carboxamide (AICAR). The synthesis of purine nucleotides requires amino acids as carbon and nitrogen donors and C_1 units are obtained from H_4 folate. IMP is the common precursor for the guanosine 5'-monophosphate (GMP) and adenosine 5'-monophosphate (AMP), with conversion to AMP and GMP requiring GTP and ATP as the energy source, respectively (Scheme 1).

Pyrimidine nucleotide synthesis

Only uridine 5'-monophosphate (UMP) is formed by the *de novo* synthesis pathway. The pyrimidine nucleotides of cytidine and thymidine are both formed from



Scheme 1. *De novo* biosynthesis of GMP and AMP. Catalyzing enzymes: [1] glutamine PRPP amidotransferase; [2] GAR synthetase; [3] GAR transformylase; [4] FGAM synthetase; [5] AIR synthetase; [6] AIR carboxylase; [7] SAICAR synthetase; [8] adenylosuccinate lyase; [9] AICAR transformylase; [10] IMP cyclohydrolase; [11] adenylosuccinate synthetase; [12] adenylosuccinase; [13] IMP dehydrogenase; [14] GMP synthetase (Cory, 1996).

uridine nucleotides, with cytidine nucleotides synthesized directly from uridine 5'-triphosphate (UTP) and thymidine 5'-monophosphate (TMP) from 2'-deoxyuridine 5'-monophosphate (dUMP).

De novo pyrimidine nucleotide synthesis

The synthesis of UMP by the *de novo* pathway involves the initial construction of the pyrimidine orotate from the amino acids glutamin and aspartate. Reaction of orotate with PRPP is catalized by the enzyme orotate phosphoribosyl transferase leading to orotidine 5'- monophosphate (OMP), which on decarboxylation gives UMP (Scheme 2).

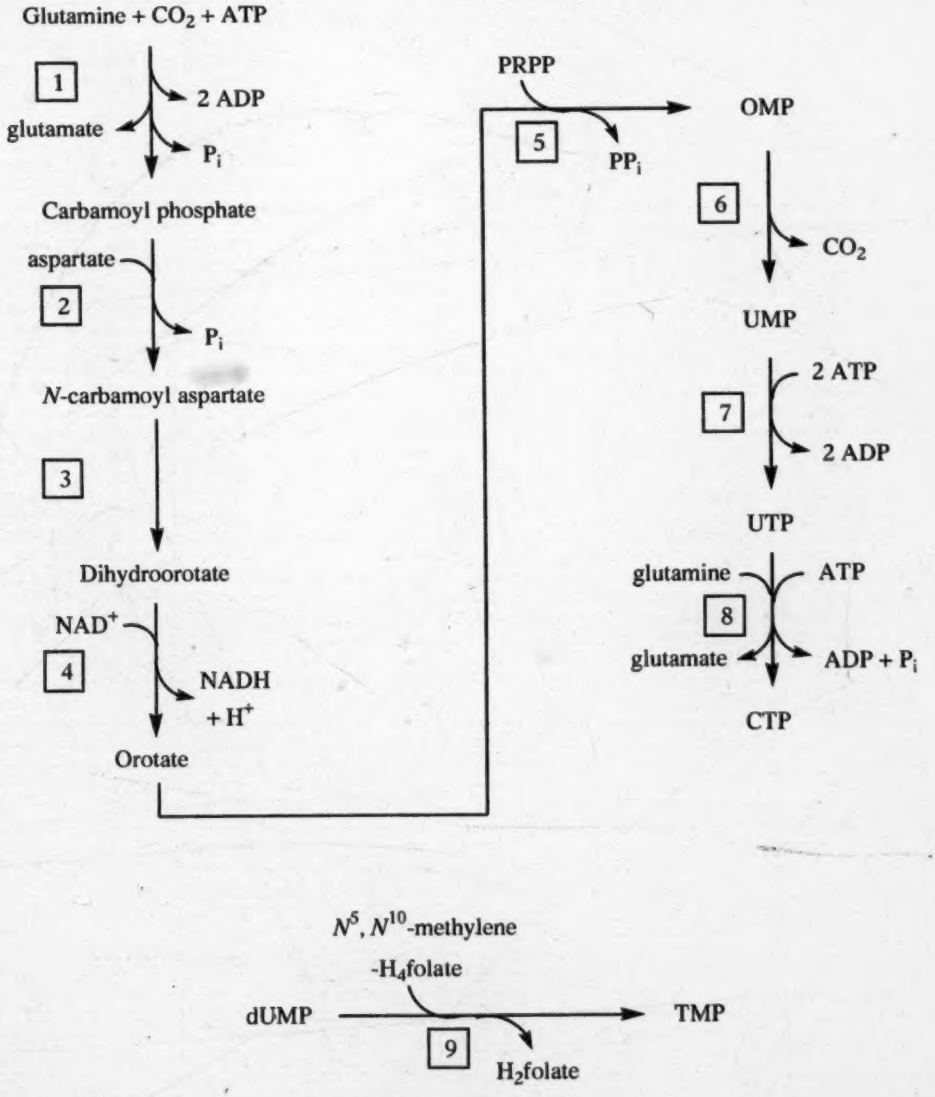
Synthesis of cytidine and thymidine nucleotides

The precursor of cytidine 5'-triphosphate (CTP) is uridine 5'-triphosphate (UTP). UMP is first converted to UTP by the action of nucleotide diphosphokinase, with CTP synthesized directly from UTP by the action of CTP synthase. The formation of thymidine 5'-monophosphate (TMP) from 2'-deoxyuridine 5'-monophosphate (dUMP) is catalyzed by thymidylate synthase (TS), which transfers a C_1 unit from N^5,N^{10}-methylene-H_4folate to dUMP. The C_1 unit is then reduced to a methyl group by the folate reactant.

Salvage pathway for nucleotide synthesis

Distinct salvage pathways exist for the purine and pyrimidine nucleoside 5'-monophosphates. Either a purine or pyrimidine base reacts with PRPP to give the

corresponding nucleoside 5'-monophosphate. Specific phosphoryl transferases (PRTase) are required to effect the salvage process. Hypoxanthine and guanine are substrates for



Scheme 2. *De novo* biosynthesis of UMP, TMP, UTP and CTP. Catalyzing enzymes: [1] carbamoyl phosphate synthetase; [2] aspartate carbamoyl transferase; [3] dihydroorotase; [4] dihydroorotate dehydrogenase; [5] orotate phosphoribosyltransferase; [6] OMP decarboxylase; [7] nucleotide diphosphokinase; [8] CTP synthetase; [9] thymidylate synthase (Cory, 1996).

hypoxanthine-guanine PRTase (HGPRTase), adenine is a substrate for adenine PRTase (APRTase), and the pyrimidines orotate, uracil and thymine are substrate for pyrimidine PRTase (PPRTase), cytosine however is not a substrate for any of the phosphoryl transferases.

Phosphorylation of nucleosides and nucleoside analogs by nucleoside kinases

Natural endogenous nucleosides must be phosphorylated to the corresponding 5'-triphosphate (TP) to be incorporated into the DNA strand being synthesized in the cell.

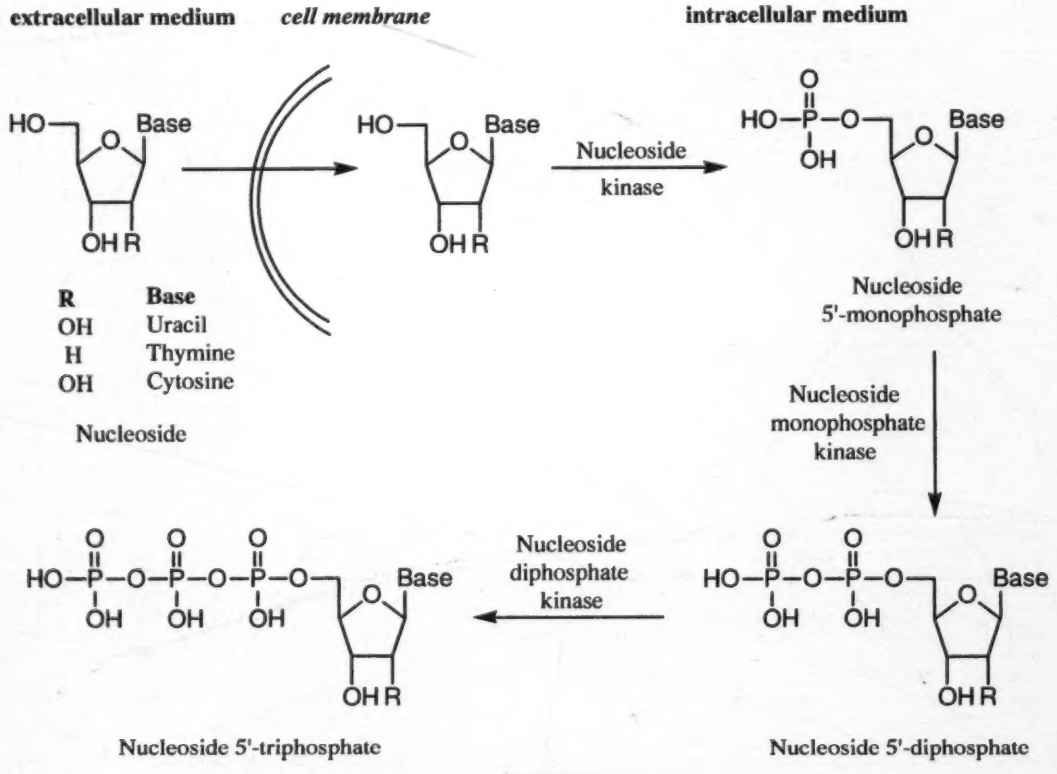


Figure 8. Intracellular metabolism of nucleosides.

The first phosphorylation step leading to formation of nucleoside 5'-monophosphate (MP), is commonly catalyzed by a nucleoside kinase encoded by the host

cell or the virus infecting the host cell (Figure 8). Conversion of nucleoside-MPs to the corresponding 5'-diphosphates (DP) and triphosphates is carried out by nucleoside monophosphate kinases (NMPKs) and nucleoside diphosphate kinases (NDPKs), respectively (Balzarini, 1993). Thus, cellular kinases and virally-encoded kinases play a vital role in the metabolism and replication of cells and viruses.

Nucleoside analogs (NAs) are used in the clinic for treatment of certain viral infections and malignant diseases. NAs are phosphorylated to their triphosphate form, and then incorporated into viral or cellular DNA by DNA polymerases or a viral transcriptase (Figure 9). Their presence in DNA causes termination of DNA elongation and often also resistance to proof-reading exonucleases. Some analogs also inhibit the reactions catalyzed by ribonucleotide reductase (RR), thymidylate synthase (TS), or 2'-deoxycytidine MP (dCMP) deaminase.

It generally is assumed that formation of the monophosphate is the rate-limiting step for metabolic activation of NAs. The human deoxyribonucleoside kinases catalyzing the initial phosphorylation step are thymidine kinase (TK) 1, deoxycytidine kinase (dCK), TK2, and deoxyguanosine kinase (dGK). dCK has been studied intensively over the years, since the enzyme is responsible for the initial activation of a number of clinically important anticancer and antiviral drugs, such as 1-(β-D-arabinofuranosyl) cytosine (Ara-C), 1-(β-D-arabinofuranosyl)adenine (Ara-A), 2-fluoro-9-(β-D-arabinifuranosyl)adenine (F-AraA), 2-chloro-2'-deoxyadenosine (CdA), 2',2'-difluorodeoxycytidine (dFdC), 2',3'-dideoxycytidine (ddC), and 2'-deoxy-3'-thiacytidine (Arner & Eriksson, 1995; Bohman *et al.*, 1990; Johansson & Karlsson, 1995). Impaired dCK expression or activity in cells lead to resistance to these drugs, indicating that dCK

plays a key role in their metabolism and pharmacological activities (Owens *et al.*, 1992). It also has been shown that sensitivity to dCK-activated NAs increases by overexpresion of dCK in tumor cell lines.

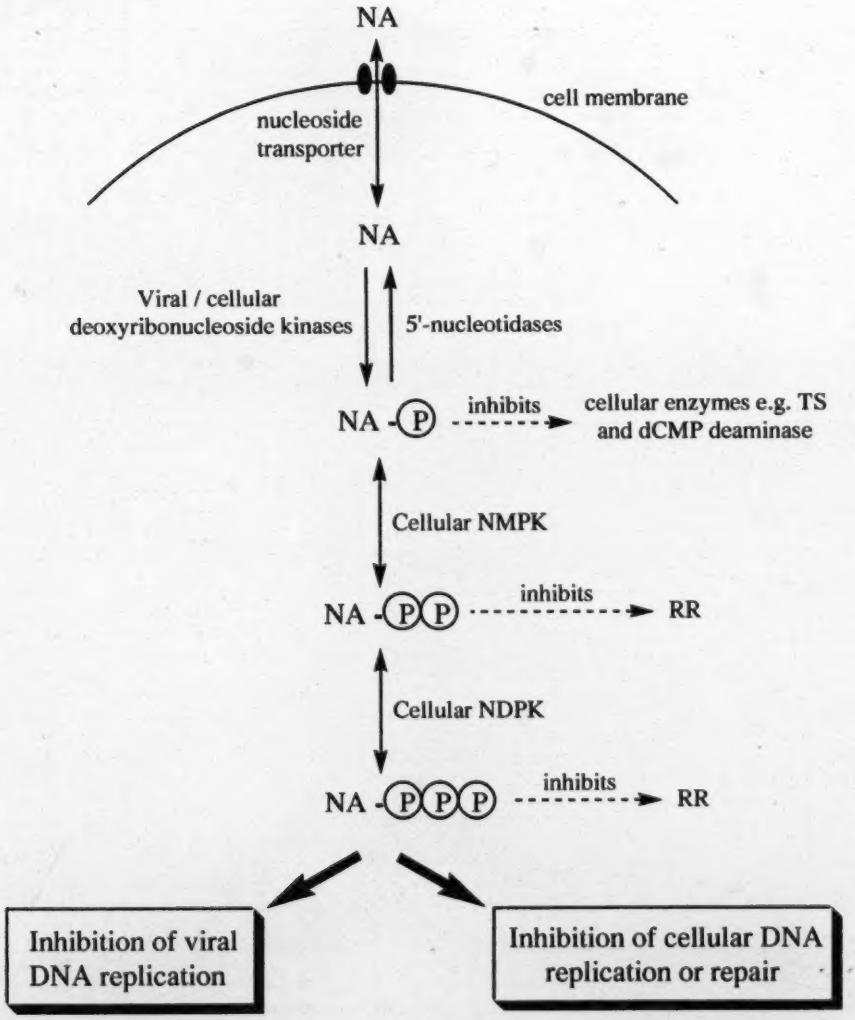


Figure 9. Nucleoside analogs require phosphorylation for pharmacological activity. The NAs are transported across the cell membrane and phosphorylated by cellular or viral enzymes to their triphosphate form (Rompay *et al.*, 2000).

Interest in dGK has increased recently since this enzyme phosphorylates a number of clinically important anticancer drugs, such as CdA, 9-β-D-arabinofuranosyl-guanine (Ara-G), 2',2'-difluorodeoxyguanosine (dFdG), F-AraA, and Ara-A (Arner & Eriksson, 1995; Sjoberg *et al.*, 1998). Zhu *et al.* (1998) showed that both *in vitro* and *in vivo* that dGK is mainly responsible for Ara-G and dFdG phosphorylation, whereas dCK is the most important enzyme for activation of CdA and dFdC. TK1 phosphorylates the pyrimidine analogs 3'-azido-2',3'-dideoxythymidine (AZT), 5-fluoro-2'-deoxyuridine (FUdR), 1-(2-deoxy-2-fluoro-β-D-arabinosyl)-5-iodouracil (FIAU), and 2',3'-didehydro-3'-deoxythymidine (Ahluwalia *et al.*, 1996; Wang & Eriksson, 1996). TK2 also phosphorylates AZT and FIAU; however, AZT is phosphorylated by TK2 at a much lower rate than by TK1, while FIAU is a better substrate for TK2 (Wang & Eriksson, 1996). dFdC and AraC are also substrates for TK2, but with a lower affinity than for dCK.

Nucleoside mimetics as anticancer and antiviral agents

Mechanisms of action of nucleoside mimetics

Nucleoside mimetics interact with either specific viral enzymes or with cellular enzymes involved in the biosynthesis of the RNA and DNA precursor nucleotides, with the resulting therapeutic effect observed dependent on the enzyme inhibited (Saunders & Cameron, 1995; De Clercq, 1994). Modification of conventional nucleosides can produce nucleoside classes with very specific enzyme targets. An increasing interest in nucleoside mimetics has led to a large variety of nucleoside structures, an ever increasing range of target enzymes and improved specificity.

1. Inhibitors targeted at reverse transcriptase

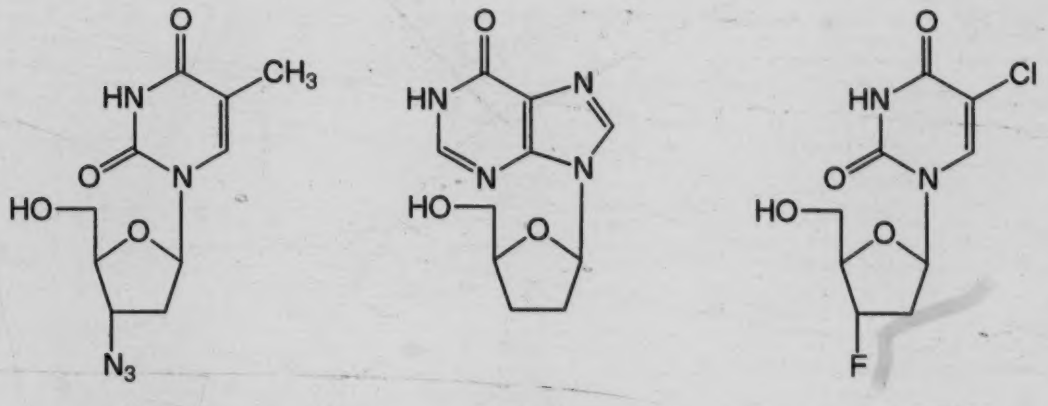
The virally encoded enzyme reverse transcriptase (RT) plays a pivotal role in the replicative process of Human Immunodeficiency Virus (HIV). RT is a multifunctional enzyme which has both RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activity as well as RNase H activity. RT is responsible for transcribing the genetic material contained within the two copies of (+)-strand RNA, located within the core of the virus, into DNA once inside the host cell. Many of the antiviral nucleoside mimetics interact at the substrate binding site of the HIV RT where they act as competitive inhibitors/alternate substrates (chain terminators) (De Clercq, 1992).

1A. Chain terminators

The first compounds approved for the treatment of AIDS were the nucleoside mimetics 3'-azido-2',3'-dideoxythymidine (AZT, Zidovudine), 2',3'-dideoxyinosine (DDI, Didanosine) and 2',3'-dideoxycytidine (DDC, Zalcytabine) (Mitsuya *et al.*,1985; Mitsuya & Broder, 1986). These 2',3'-dideoxynucleosides (DDN's) all lack a 3'-hydroxy group and are active in their triphosphate forms (DDNTP). These DDNTP analogues interact at the substrate (dNTP) binding site of the HIV RT where they act as competitive inhibitors/alternate substrates. A 3'-hydroxy group is essential for extension of the viral DNA chain, therefore once incorporated into the growing DNA chain, DDNTPs inhibit the action of RT by premature chain termination. Some of the antiviral nucleoside mimetics which exhibit this mechanism of action are shown in Figure 10.

The DDN's show differences in their ability to suppress HIV, and this has been shown to be directly related to their intracellular metabolism to the 5'-triphosphate form

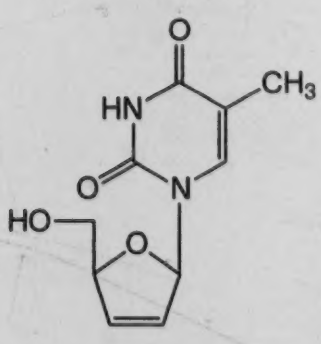
(Balzarini *et al.*, 1989). For stavudine (D_4T) the rate-limiting step is the formation of D_4T-MP. However, for AZT the rate-limiting step is the formation of AZT-DP from AZT-MP, this is attributed to the ability of AZT-MP to inhibit dTMP kinase, the enzyme which catalyses the conversion of AZT-MP to AZT-DP.



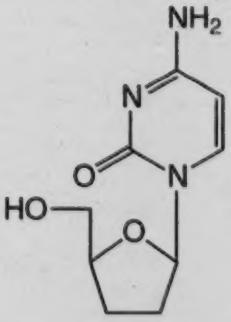
AZT
(Zidovudine)

DDI
(Didanosine)

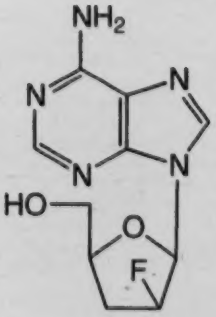
FddClU



D4T
(Stavudine)



DDC
(Zalcytabine)



FddAraA

Figure 10. Alternate substrates/chain terminators.

1B. HIV-1 specific reverse transcryptase inhibitors

Two compounds of particular interest are 1-(2-hydroxyethoxymethyl)-6-phenylthiothymidine (HEPT) (Miyasaka *et al.*, 1989) and 2',5'-bis-O-(*tert*-

butyldimethylsilyl)-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide)thymidine (TASO-T) (Balzarini *et al.*, 1992) (Figure 11).

These compounds are specific inhibitors of HIV-1 RT and do not affect the replication of other retroviruses. HEPT and TSAO-T are described as non-nucleoside RT inhibitors (NNRTI's) as they do not bind at the substrate (dNTP) binding site. The selectivity of the NNRTI's is attributed to a specific interaction with a non-substrate site of the HIV-1 RT.

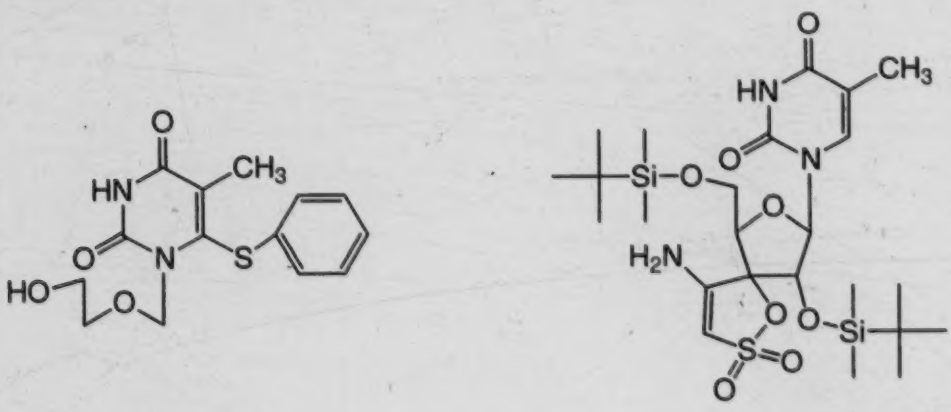


Figure 11. Specific HIV-1 RT inhibitors.

2. Inhibitors targeted at DNA viruses

The mechanism of action of nucleoside mimetics which act as inhibitors of DNA viruses such as Hepatitis B virus (HBV), Herpes Simplex virus (HSV), Epstein-Barr virus (EBV), Varicella Zoster virus (VZV) and Cytomegalovirus (CMV), varies depending on the structure class to which nucleoside mimetic belongs. These mimetics either act as inhibitors/substrates of the viral DNA polymerase, resulting in chain termination, or DNA strand breakage.

2A. DNA polymerase inhibitors/chain terminators

The acyclic nucleosides acyclovir (ACV, Zovirax) and ganciclovir (GCV, Cymevene) and the carbocyclic nucleosides penciclovir (PCV, Vectavir) and carbaoxetanocin G (BHCG, Lobucavir) (Figure 12) are recognized as substrates by the HSV- and VZV-encoded thymidine kinase (TK) (De Clercq, 1994).

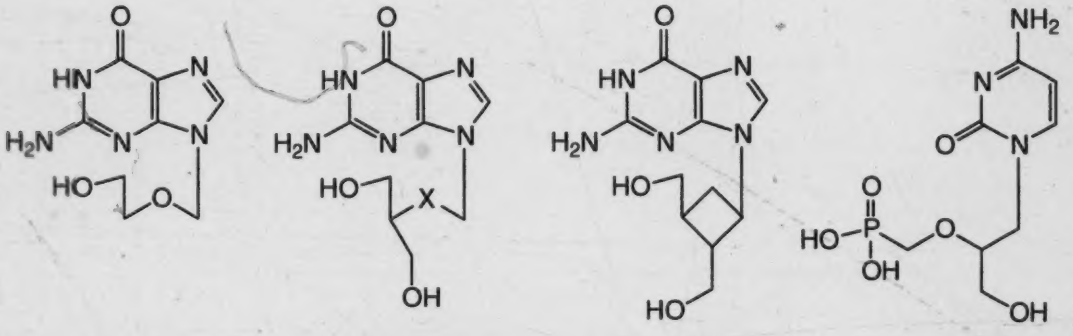


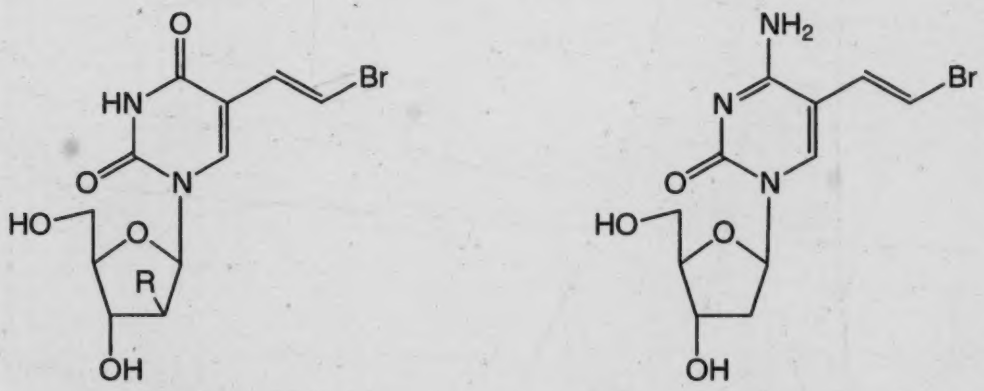
Figure 12. Viral DNA chain terminators.

The viral TK converts these mimetics to their monophosphates, and then cellular enzymes convert to the di- and then tri-phosphates, which is the required form for activity. The triphosphates act as substrates of the viral DNA polymerase and after incorporation into the viral DNA, chain termination occurs. The acyclic nucleoside phosphates can also act in this manner. Cidofovir (HPMPC) (Figure 12) has good activity against CMV and is active as its diphosphate HPMPC-DP (Ho *et al.*, 1992). HPMPC-DP may act as a competitive inhibitor/substrate of viral DNA polymerase being incorporated either terminally, and so causing chain termination, or internally resulting in the

formation of aberrant DNA, the overall effect being the impaired synthesis of the viral DNA.

2B. Viral DNA strand breakage

(*E*)-5-(2-Bromovinyl)-2'-deoxyuridine (BVDU) and its analogues are substrates for HSV-1 TK and VZV-TK (Figure 13), this specificity is attributed to the (*E*)-5-(2-bromovinyl) substituent (De Clercq *et al.*, 1986). BVDU is converted to the diphosphate by either HSV-1 TK or VZV-TK and then to its active triphosphate form by cellular kinases. The triphosphate acts as a substrate of the viral DNA polymerase and is incorporated into the interior of the DNA chain, which results in DNA strand breakage (Balzarini *et al.*, 1990).



R = H, **BVDU**
R = OH, **BVaraU**

BVDC

Figure 13. Viral DNA strand breakers.

3. Inhibitors targeted at nucleotide metabolizing enzymes

Nucleotide metabolizing agents play an important role in the cell cycle and the rate of cell division. During the cell cycle, all the components of the cell double and

eventually cell division occurs leading to the formation of two daughter cells. RNA synthesis and DNA replication occur at different stages of the cell cycle, with RNA synthesis occurring during G_1, S and G_2 phases of the cell cycle and DNA replication in S phase. The levels of these nucleotide metabolizing enzymes are elevated further in rapidly growing cells such as tumor cells, therefore inhibition of these enzymes by nucleoside mimetics can be beneficial in cancer therapy as well as antiviral therapy.

3A. Inhibitors of purine nucleotide metabolizing enzymes

The main enzymatic targets involved in purine biosynthesis are inosine monophosphate dehydrogenase (IMP dehydrogenase, IMPDH), purine nucleoside phosphorylase (PNP) and *S*-adenosyl-L-homocysteine (AdoHcy, SAH) hydrolase. Some of the nucleoside mimetics which inhibit these key enzymes are shown in Figure 14.

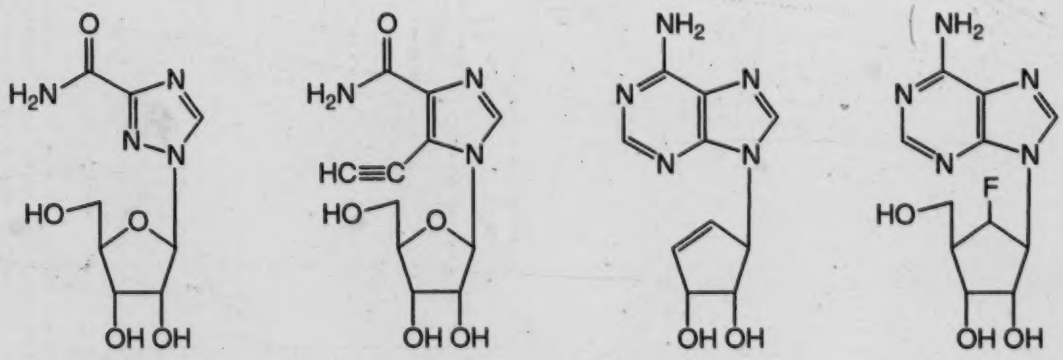
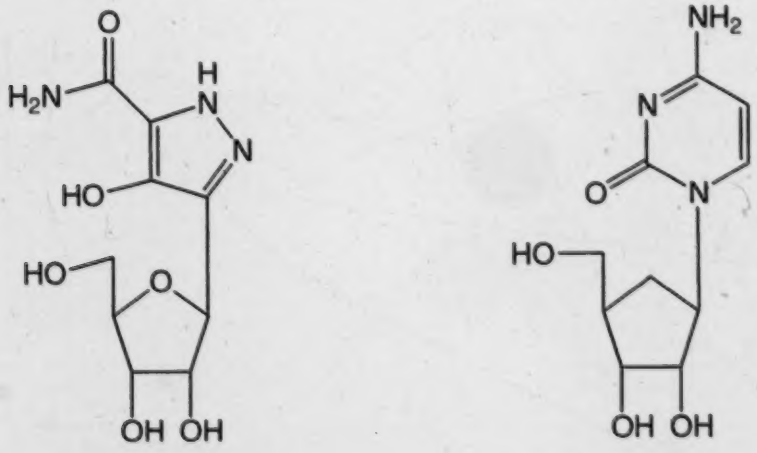


Figure 14. Inhibitors of IMP dehydrogenase, PNP and AdoHcy hydrolase.

3B. Inhibitors of pyrimidine nucleotide metabolizing enzymes

OMP decarboxylase which catalyses the final step in the biosynthesis of UMP, and CTP synthetase which converts UTP to CTP, are two targets in the biosynthesis of pyrimidine nucleotides. The OMP decarboxylase inhibitor pyrazofurin (Figure 15), which is active against a broad spectrum of viruses including influenza A, B, C (Shannon, 1977) and murine leukemia, (Shigeta *et al.*, 1988), causes cessation of the synthesis of all pyrimidines resulting in impaired cell growth and metabolism. Cyclopentylcytosine (C-Cyd) (Figure 15), which is active in its triphosphate form, is selective in targeting CTP synthase (De Clercq *et al.*, 1990). Inhibition of CTP synthase causes a depletion of CTP, CDP, dCDP and dCTP pools.



Pyrazofurin **C-Cyd**

Figure 15. Inhibitors of OMP decarboxylase and CTP synthase.

4. Inhibitors of thymidylate synthase

A critical step in the *de novo* pathway of DNA synthesis is the production of the pyrimidine nucleotide thymidine 5'-monophosphate (TMP) from 2'-deoxyuridine-5'-monophosphate (dUMP). This reaction is catalyzed by the enzyme thymidylate synthase

(TS) using the folate cosubstrate 5,10-methylene tetrahydrofolate (CH_2THF) and is the only source of *de novo* cellular thymidylate. TS is a two-substrate enzyme that accepts both dUMP and CH_2THF to form a ternary complex through which reductive methylation of dUMP to TMP occurs. In this process, tetrahydrofolate is oxidized to dihydrofolate (Figure 16).

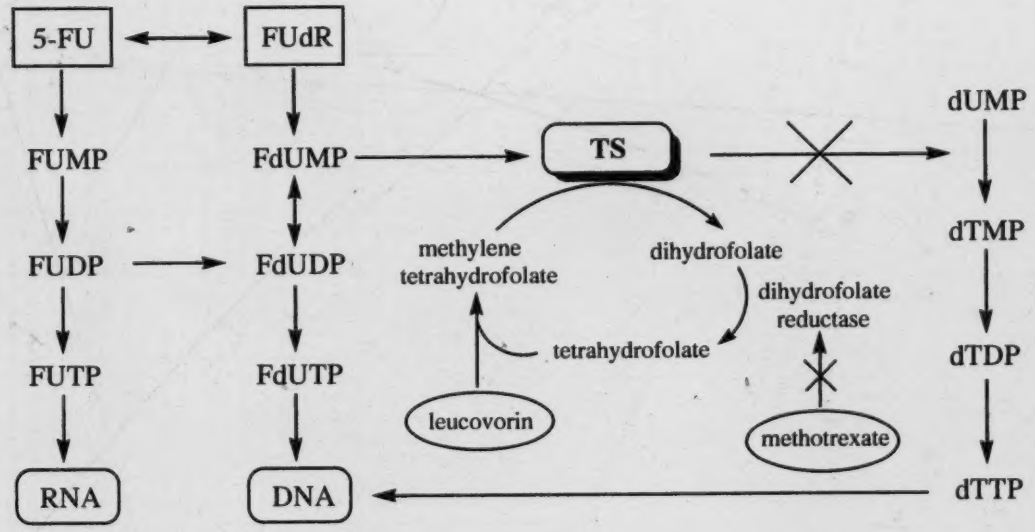
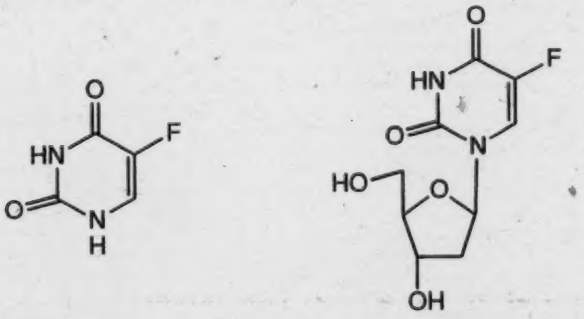


Figure 16. Mechanism of action of 5-FU and FUdR and the influence of the modulators leucovorin and methotrexate. Both 5-FU and FUdR can after appropriate metabolic conversion, be incorporated in the DNA and RNA and have detrimental effects on those nucleic acids. Both drugs can be metabolized into FdUMP that is a strong inhibitor of the key enzyme in DNA synthesis, thymidylate synthase (TS) (Schuetz *et al.*, 1984).

This reaction provides the *de novo* cellular thymidylate nucleotides necessary for DNA synthesis and repair. TS inhibition results in a thymidine-less state, which is toxic to actively dividing cells. This cytotoxicity is likely effected by DNA fragmentation that results from thymidine 5'-triphosphate (dTTP) depletion, which increases

misincorporation of dUTP (Ayusawa *et al.*, 1988). The enzyme involved in regeneration of tetrahydrofolate from dihydrofolate is dihydrofolate reductase (DHFR). Folate analogs (antifolates), such as methotrexate (MTX), inhibit DHFR, deplete tetrahydrofolate, and cause a marked decrease in pyrimidine and purine synthesis (Harrap *et al.*, 1989).



5-FU **FUdR**

Figure 17. Chemical structure of the fluoropyrimidines 5-FU and FUdR.

TS inhibition can be achieved with either fluoropyrimidines or folate analog compounds. The first compounds to have clinically significant TS-inhibiting activity were 5-fluorouracil (5-FU) and 5-fluoro-2'-deoxyuridine (FUdR) (Figure 17); these are metabolized to 5-fluoro-2'-deoxyuridylate (the most efficacious analog of deoxyuridylate) and form a ternary complex together with TS and CH_2THF, resulting in potent TS inhibition. These drugs may also become incorporated into RNA or DNA via fluoro-UTP (Heidelberger, 1965) or 5-fluoro-dUTP (Schuetz *et al.*, 1984), respectively (Figure 16). A family of compounds, the halogen-substituted 5-vinylic deoxyuridines such as BVDU, has been studied extensively as antiviral agents (De Clercq, 1997). These compounds have been characterized as requiring herpesvirus-encoded thymidine kinase for monophosphorylation in human cells; they are proposed to then react with TS,

resulting in active site modification and inactivation of the enzyme (Balzarini *et al.*, 1987).

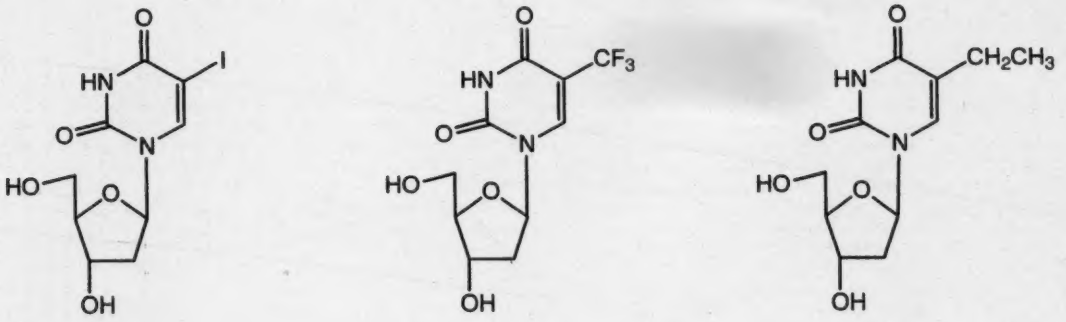
Selective antiherpesvirus agents

Shortly after it had been described as a potent and selective inhibitor of the replication of HSV and, to a lesser extent, of varicella-zoster virus (VZV), acyclovir became the drug of choice for the treatment of HSV and VZV infections, particularly primary and recurrent genital herpes and mucocutaneous HSV and VZV infections in immunosuppressed patients. Also, acyclovir proved to be superior to vidarabine in the treatment of herpetic encephalitis and VZV infections and has replaced vidarabine for these indications (De Clercq, 1993). Because of its limited oral bioavailability (only 20%), acyclovir has, in turn, been replaced by its prodrug, valaciclovir, in the oral treatment of HSV and VZV infections (Crooks & Murray, 1994).

The remarkable potency of acyclovir against HSV-1, HSV-2, and to a lesser extent VZV has prompted the development of several structural analogs of acyclovir. Foremost among these acyclovir congeners are ganciclovir and penciclovir. Ganciclovir has a more pronounced activity against cytomegalovirus (CMV) than acyclovir and became the drug of choice (until the advent of HPMPC) for the treatment of CMV infections, particularly CMV retinitis, in immunosuppressed patients (Markham *et al.*, 1994). Penciclovir has a similar activity spectrum and mechanism of action to acyclovir and has been used under its oral prodrug form, famciclovir, for the same indications as valaciclovir, i.e., for the treatment of HSV and VZV infections (Vere Hodge, 1993). In addition, penciclovir shows activity against human hepatitis B virus.

Whereas acyclovir emanated from a program centered on examining the substrate specificity of adenosine deaminase (which is known to rapidly convert the antiviral compound vidarabine [ara-A, adenine arabinoside, 9-β-D-arabinofuranosyladenine] to its inactive metabolite ara-Hx [hypoxanthine arabinoside]), brivudin [(*E*)-5-(2-bromovinyl) 2'-deoxyuridine, BVDU] was originally developed at the Chemistry Department of the University of Birmingham as a potential radiation-sensitizing agent (assuming that it would be incorporated into DNA). Like acyclovir, BVDU turned out to be a potent and selective anti-HSV agent (De Clercq *et al.*, 1979). Its potency and selectivity against HSV-1, BVDU exceeded all other anti-HSV agents, including acyclovir and the older 5-substituted 2'-deoxyuridines such as IUdR (5-iodo-2'-deoxyuridine), TFT (5-trifluoromethyl-2'-deoxyuridine), and EDU (5-ethyl-2'-deoxyuridine) (De Clercq, 1985) (Figure 18).

The 2(*E*)-bromovinyl group, with the bromine in the *trans* (or *Entgegen*) configuration, is crucial for the antiviral selectivity of BVDU, and hence various other compounds that have been synthesized since BVDU and that share with BVDU the same 5-2(*E*)- bromovinyl substituent demonstrate similar selectivity, potency, and activity spectrum to those of BVDU; these include BVaraU (sorivudine) (Machida *et al.*, 1981), C-BVDU (carbocyclic BVDU) (Herdewijin *et al.*, 1985), and S-BVDU (4'-thio-BVDU) (Figure 18) (Walker, 1997). Also, 5-(5-bromothien-2-yl) 2'-deoxyuridine (BTDU) (Figure 18), containing a 5-(5-bromothien-2-yl) substituent with a bromine group in roughly the same position as in BVDU, exhibits similar selectivity and potency to BVDU (Wigerinck *et al.*, 1991).



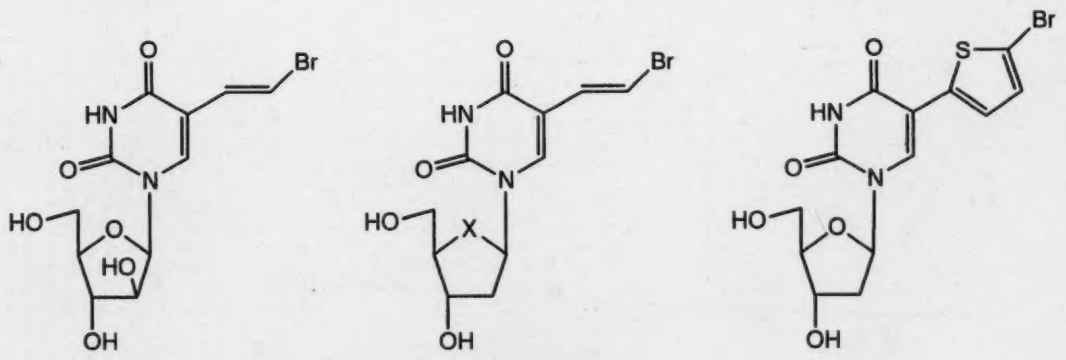


Figure 18. 5-substituted 2'-deoxyuridines.

The mechanism of action of BVDU (Figure 19) depends on a specific phosphorylation by the virus-encoded TK, i.e., the TKs induced by HSV-1 and VZV, which convert the compound to its 5'-monophosphate (BVDU-MP) and 5'-diphosphate (BVDU-DP) (Ayisi *et al.*, 1984; Descamps & De Clercq, 1981). Upon further phosphorylation by cellular kinase(s), i.e., nucleoside 5'-diphosphate (NDP) kinase, BVDU 5'-triphosphate (BVDU-TP) can act in a dual fashion with the viral DNA polymerase: (i) as a competitive inhibitor with respect to the natural substrate (dTTP) (Allaudeen *et al.*, 1981) or (ii) as an alternative substrate, which would then allow

BVDU-TP to be incorporated (as BVDU-TP) into the DNA chain. This incorporation may, in turn, affect both the stability and the functioning of the DNA.

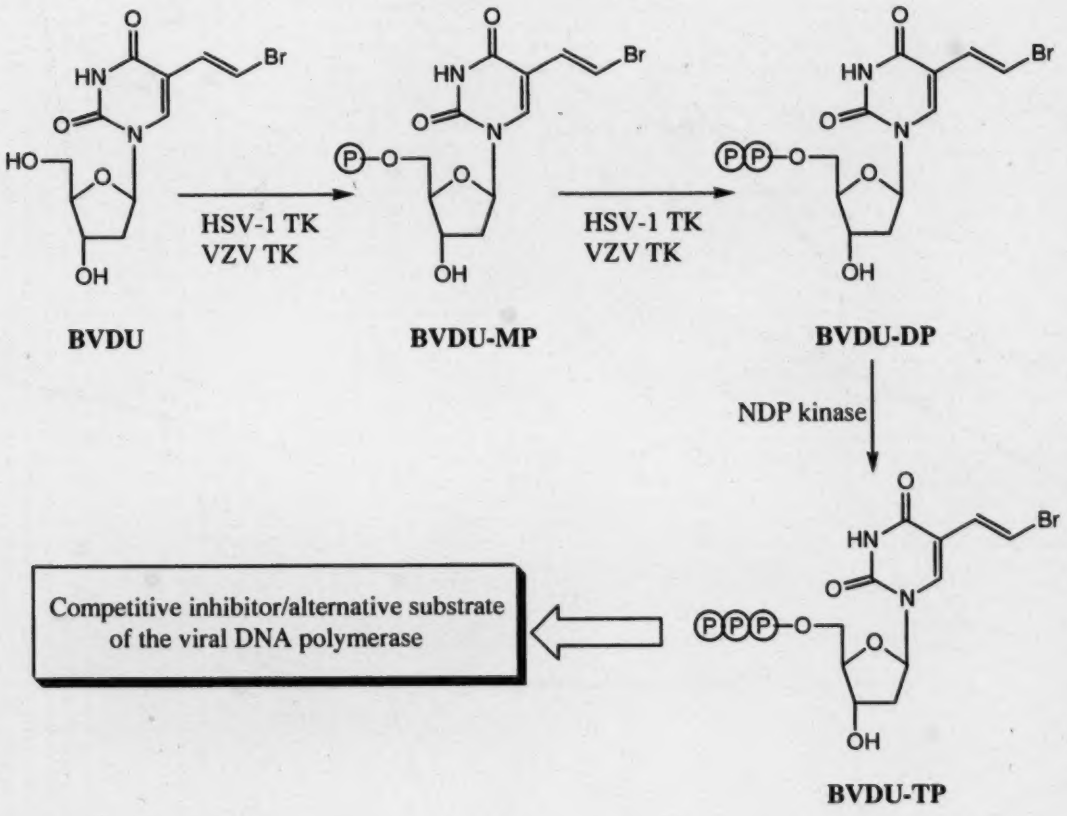


Figure 19. Mechanism of action of BVDU. Following uptake by the cells and intracellular phosphorylation by the virus-encoded TK to the 5'-monophosphate (BVDU-MP) and the 5'-diphosphate (BVDU-DP) and further phosphorylation (presumably by the NDP kinase) to the 5'-triphosphate (BVDU-TP), the last compound acts as a competitive inhibitor/alternative substrate of the viral DNA polymerase and can be incorporated internally (via internucleotide linkage) into the DNA chain.

BVDU displays a remarkable specificity in its antiviral activity spectrum: it is a highly potent inhibitor of HSV-1 but not HSV-2, so that it can be used as a marker for

differentiating HSV-2 from HSV-1 strains (De Clercq, 1984). The reason for the relative inactivity of BVDU against HSV-2 is that the HSV-2-encoded TK is unable to phosphorylate BVDU- MP to BVDU-DP. This results in a substantial reduction in the supply of the active BVDU metabolite, BVDU-TP, in the HSV-2-infected cells (Ayisi *et al.*, 1984). BVDU and its arabinofuranosyl counterpart BVaraU belong to the most potent inhibitors of VZV that have ever been described. At nanomolar concentrations, they inhibit the replication of VZV (Andrei *et al.*, 1995). This is in marked contrast to the established anti-VZV drugs, acyclovir and penciclovir, whose inhibition of VZV replication takes place only at micromolar concentrations.

BVDU is also a potent inhibitor of the replication of Epstein-Barr virus (EBV) in vitro (Lin *et al.*, 1983), but not CMV or human herpesvirus 6 (HHV-6) and 7 (HHV-7) (Reymen *et al.*, 1995). In addition, BVDU has been found effective against a number of herpesviruses of veterinary importance such as swine herpesvirus type 1 (SHV-1), bovine herpesvirus type 1 (BHV- 1), simian varicella virus (SVV), and herpesvirus platyrrhinae (HVP) but not equine herpesvirus type 1 (EHV-1) (De Clercq, 1984). As for HSV-2, the insensitivity of EHV-1 to BVDU could be attributed to an inefficient phosphorylation of BVDU-MP to BVDU-DP in the virus-infected cells (Kit *et al.*, 1987).

Due to its unique propensity for some virus-encoded TKs, BVDU could also be used as a cytostatic agent, provided that the tumor cells have been transfected by the virus-specific TK. Thus, murine mammary FM3A carcinoma cells transformed with the HSV-1 TK gene are inhibited in their growth by BVDU at 1 ng/mL, a 10,000-fold-lower concentration than that required to inhibit the untransformed cancer cells (Balzarini *et al.*, 1985). The cytostatic activity of BVDU against cancer cells that have been transfected by

the HSV-1 (or HSV-2) TK gene is due to the inhibitory effect of BVDU-MP (formed intracellularly by the viral TK) on the cellular thymidylate synthase (Balzarini *et al.*, 1993). In addition to BVDU, *S*-BVDU and BTDU (but not BVaraU) (Figure 18) can serve as HSV TK-activated cytostatic agents targeted at the thymidylate synthase (Bohman *et al.*, 1994). BVDU and its congeners *S*-BVDU and BTDU should therefore be considered potential candidate drugs for the treatment of HSV TK gene-transfected tumors.

Pronucleotides for the *in vivo* delivery of antiviral and anticancer nucleotides

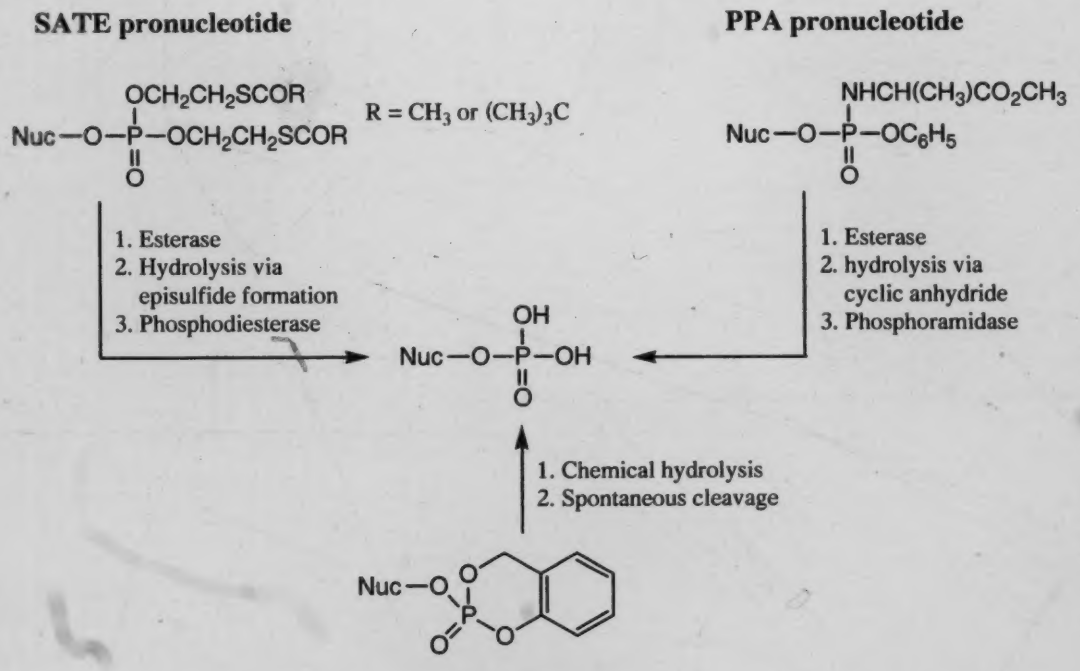
Nucleoside analogs have been a subject of numerous studies as antiviral and anticancer agents. In order to be active they must be transformed to nucleotide metabolites by viral and/or cellular enzymes. This activation follows the pathway utilized by natural nucleosides. Based on the metabolite-antimetabolite approach, nucleoside analogs such as 2',3'-dideoxynucleosides (ddNs) have been developed as competitors of natural 2'-deoxynucleoside 5'-triphosphates (dNTPs). Typically, modifications at the 2' or the 3'carbon atoms of the glycone (sugar) moiety of nucleosides are introduced. By virtue of their resemblance to the natural 2'-deoxynucleosides, ddNs are phosphorylated to the corresponding 5'-triphosphates and incorporated into a growing DNA strand by a DNA polymerase, resulting in chain termination. Therefore, ddNs are in essence prodrugs, since they must be phosphorylated intracellularly in order to be biologically active. Therapies involving long-term administration of ddNs such as 3'-azido-2',3'-dideoxythymidine (AZT) have been reported to lead to decreased activity of the first phosphorylating enzyme, thymidine kinase and thus, resistance (Antonelli *et al.*, 1996).

This type of resistance is observed not only in host tissues of the patients undergoing ddN therapy, but also in viruses. This resistance mechanism renders the ddNs less effective since their activation is hindered at the first phosphorylation step. In addition, for antiviral acyclic nucleosides such as acyclovir and penciclovir, the dependence of the nucleosides on activation to the triphosphates by virally encoded thymidine kinase, limits their spectrum of antiviral activity to those viruses such as herpes simplex virus (HSV) and Varicella zoster virus (VZV) which encode their own thymidine kinase (Elion, 1983). Thus, viruses such as hepatitis B virus (HBV), which do not encode their own nucleoside kinase, do not fall within the purview of activity of these antiviral nucleosides. Moreover, ddNs such as AZT, are associated with myelosuppressive side effects, such as anemia and neutropenia (Sommadossi, 1992). Toxic side effects have been widely reported to lead to the discontinuation of ddN therapy. Certain other ddNs such as 2',3'-dideoxyuridine (ddU), are poor substrates for thymidine kinase or other cellular kinases, and are, therefore, not converted to the corresponding triphosphates (Sastry *et al.*, 1992; Gosselin *et al.*, 1996).

In principle, administration of the 5'-phosphates would aid in overcoming the drawbacks of ddN therapy posed by resistance mechanisms and inherent biological differences. However, because phosphates are strongly acidic and thus negatively charged at physiological pH (pH = 7.4), they are too hydrophilic to penetrate the lipid-rich cell membranes. In addition, blood and cell surface phosphohydrolases (acid and alkaline phosphatases, 5'-nucleotidases) rapidly convert the phosphates to the corresponding nucleosides.

In order to overcome the poor cell penetration of nucleoside 5'-phosphates, Montgomery *et al.* (1961) proposed that "this difficulty might be overcome if one could prepare an ester of a nucleotide which could penetrate the cell wall and then be metabolized to the nucleotide itself." Consequently, various prodrug or "pronucleotide" (Gosselin *et al.*, 1996) approaches have been devised and investigated. In general, the goal of these approaches has been to promote passive diffusion through cell membranes and increase the bioavailability of phosphorylated nucleosides. This approach of derivatization has been applied using various protecting groups for the phosphate moiety. Ideally, the attempts have been designed to achieve stability in the extracellular medium and rapid intracellular hydrolysis to release the phosphate. In most cases, to be biologically active, the phosphate has to be activated to the diphosphate and triphosphate. Three groups of pronucleotides, each with a different mechanism of action, have gained prominence as antiviral agents effective primarily against HIV (Scheme 3). The *S*-acylthioethylphosphate (SATE) pronucleotides elaborated by Perigaud *et al.* (1999), the phosphoramidates derived from amino acids introduced by McGuigan *et al.* (1996) and, finally, cyclosaligenyl (cycloSal) pronucleotides designed and studied by Meier (1998). In the case of amino acid phosphoramidates, the studies have been largely focussed on phenyl phosphoralaninates (PPA) of anti-HIV agents zidovudine (AZT) and stavudine (d4T). Extensive structure.activity relationship studies (McGuigan *et al.*, 1996; Kinchington *et al.*, 1992) indicated that a combination of alanine ester amidate and phenyl phosphate provides for the most effective agents. Consequently, PPA triesters have been used by several groups of investigators as antiviral pronucleotides (Franchetti *et al.*, 1994; Winter *et al.*, 1996), and potential antitumor agents (Manfredini *et al.*, 1999;

Kumar *et al.*, 2001). In stereochemical terms, only SATE pronucleotides do not contain a chiral phosphorus atom but both cycloSal and amidate prodrugs (alanine ester has an L-configuration) derived from natural (D) nucleosides are mixtures of two diastereoisomers.



Scheme 3. Pronucleotide approach (Nuc = nucleoside-5' residue).

C-Nucleosides

The unique structural characteristic of *C*-nucleosides which distinguishes them from the native *N*-nucleosides is the presence of a carbon to carbon bond instead of a carbon to nitrogen bond between the aglycone and the sugar moieties. Although *N*-nucleosides which have a C1'-N glycosidic link predominate in nature, there are many naturally occurring *C*-nucleosides which have a C1'-C glycosidic link (Shaban & Nasr,

1997; Shaban, 1998; Knutsen, 1992). The first *C*-nucleoside isolated was pseudouridine (Cohn & Volkin, 1951), many other members of this class of nucleoside have now been isolated from natural sources, such as showdomycin (Daves & Cheng, 1976), formycin (Hori *et al.*, 1968), pyrazofurin (Gerzon *et al.*, 1971) and oxazinomycin (Haneishi *et al.*, 1971), or prepared synthetically such as tiazofurin (Fuertes *et al.*, 1976) and selenazofurin (Srivastava & Robins, 1983) (Figure 20).

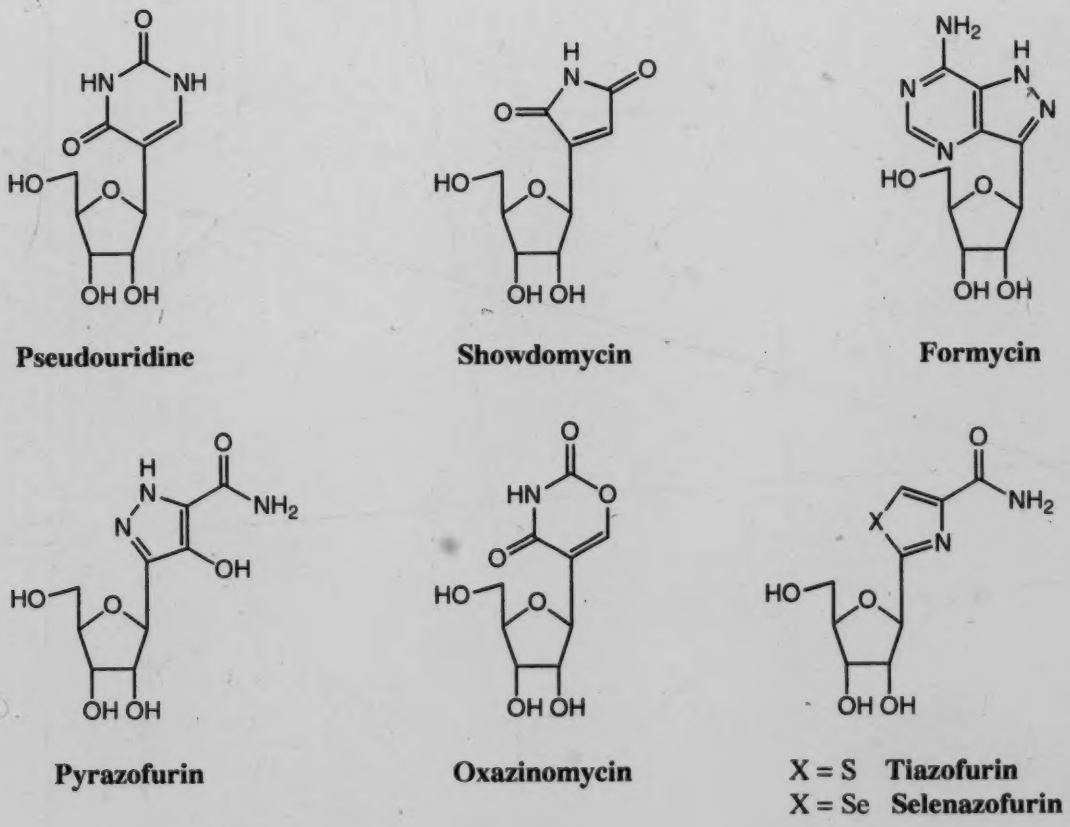


Figure 20. Examples of natural and synthetic *C*-nucleosides.

C-nucleosides act as isosteric mimics of the *N*-nucleoside metabolites and as such often display antibacterial, antiviral and antitumor properties (Shaban & Nasr, 1997;

Shaban, 1998; Knutsen, 1992). Importantly, the C-C glycosidic link renders *C*-nucleosides stable to enzymatic hydrolysis.

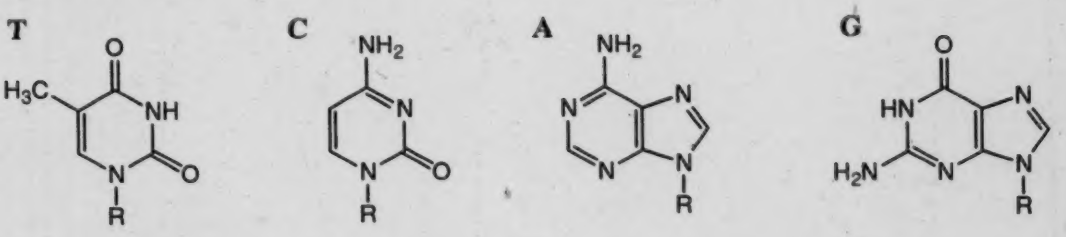
_C_-Arylglycosides as nucleoside mimetics

C-arylglycosides (or non-nitrogen heterocyclic *C*-nucleosides) are widespread in nature and extremely diverse with respect to the aglycone moiety. The chemistry, biochemistry and occurrence of *C*-arylglycosides have been the subject of several reviews (Postema, 1992; Hanessian & Pernet, 1976; Franz & Grun, 1983).

More recently, the use of *C*-arylglycosides as nonpolar isosteres of natural nucleosides has been reported by Kool *et al.* (1997) with surprising results. The interest in nonpolar isosteres of nucleosides was to study non-covalent interactions involving DNA and RNA, such as molecular recognition and catalytic events. These isosteres would be required to closely mimic the size and shape of the natural nucleosides but lack any significant hydrogen bonding capability. Therefore, substituted benzenes were prepared as mimetics of pyrimidine nucleosides and indoles as mimetics of the purine nucleosides (Figure 21) (Schweitzer & Kool, 1994).

Unexpectedly, studies involving DNA template strands containing the thymidine mimetic difluorotoluene nucleoside (Figure 21) showed that it was an excellent and highly specific template for replication (Guckian & Kool, 1997). Despite its low hydrogen bonding capability, the enzyme *E. coli* DNA polymerase I recognized the isostere as thymidine. The results obtained from this research suggested that steric factors rather than hydrogen bonding may be the more important factor in replication.

Natural Nucleosides



Nonpolar Isosteres

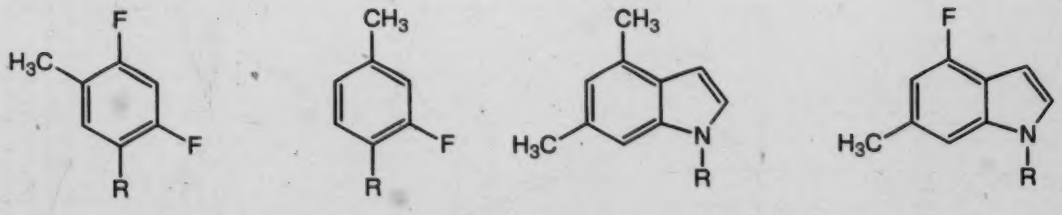


Figure 21. Non-polar isosteres of natural nucleosides (R = deoxyribose).

Gene therapy of cancer

Gene therapy is essentially the transfer of nucleic acids (DNA, RNA) into target cells to correct or disrupt a pathologic process. There are two well-defined approaches:

- *in vitro gene therapy*: the transfer of genes into cells cultured outside of the body prior to placing those cells back into the body, and
- *in vivo gene therapy:* the direct administration of a gene, or genes, to reach a target cell type in the body.

The idealized approach to gene therapy is the replacement of a mutated gene with a corrected copy that restores normal funtion and therapeutically alters the deleterious phenotype. To date, three separate strategies have been pursued (Holland, 2000; Hall *et al.*, 1997):

1. Replacement and knockout gene therapy
2. Suicide gene therapy
3. Immunomodulatory gene therapy

Replacement and knockout gene therapy

Replacement gene therapy is a method for replacing a mutated or missing gene (usually a tumor suppressor gene) that serves to keep cell growth and division under control with a "healthy" (that is, normal) copy of that gene. The goals of tumor suppressor gene therapy include the induction of cell death (apoptosis), and the production of changes in cell growth, behavior, invasiveness, or metastatic ability. Since *p53* is the most commonly mutated gene in human cancer that influences transcription, cell-cycle arrest, DNA repair, and apoptosis as well as angiogenesis (the development of a tumor's blood supply), it has received the most interest as a target for cancer gene therapy. Transduction (the delivery or direct transfer of genetic material from one cell to another) of cancer cells with *p53* can significantly inhibit growth and angiogenesis or can induce apoptosis in *p53* mutant cells in several tumor models, including lung and breast. The potential efficacy of replacement gene therapy with tumor suppressor genes is still limited by the large number of target genes clearly known to induce or maintain malignancy, and the difficulty of transducing enough cancer cells to result in a cure.

Knockout gene therapy primarily targets the products of oncogenes in an effort to inactivate them and reduce cellular proliferation. Several approaches are being tried:

- delivering (transduction) of a mutant oncogene that acts in a *dominant* manner to negate the effects of the cancer-causing oncogene;

- inhibiting translation of the oncogene by inserting DNA that codes for the production of a specialized RNA that will destroy the oncogene's RNA; and
- reducing the levels of oncogene product that are being made in the cell by delivering an *antisense mRNA* that binds to the mRNA produced by the oncogene in order to interfere with the synthesis of the oncogene protein product or make the bound oncogene's mRNA susceptible to breakdown by normal cellular enzymes (nucleases).

While replacement and knockout gene therapy has had its successes in slowing the growth of cancer cells in the laboratory, it will take multiple replacements of different genes to arrest the growth of tumors. This is because multiple genetic aberrations are usually involved in creating and maintaining cancerous transformation of cells. Also, an unexpected *bystander effect*—the death of more cells than are actually transduced—has been observed and this effect may result from cell-cell contact, immune-mediated responses, and/or other local actions.

Suicide gene therapy

Suicide gene therapy—also referred to as the use of chemosensitization genes—involves the transduction of a gene that transforms a nontoxic form of a drug (that is, a "pro-drug") into a toxic substance. Two examples illustrate the nature of this strategy:

1. *The E. coli cytosine deaminase gene (CD) plus 5-fluorocytosine*: CD converts 5-fluorocytosine to 5-fluorouracil, a chemotherapeutic agent. This combination produces a bystander effect and has been demonstrated to have some success in animals with hepatic metastases of gastrointestinal tumors.

2. *The herpes simplex virus thymidine kinase gene (HSV-*tk*) plus nucleoside analogs, such as acyclovir or ganciclovir*: The *tk* gene can modify a variety of nucleoside analogs by phosphorylation. When this occurs, followed by incorporation of the phosphorylated analog into replicating DNA, the elongation of the replicating DNA strand is stopped and cell death follows. Like the *E. coli* CD system, this also causes a bystander effect—not every cell within a tumor has to be transduced by the *tk* gene in order to wipe out the tumor. This strategy has been evaluated for possible treatment of localized brain tumors, liver metastases, peritoneal-based metastases, and mesotheliomas.

Additional suicide genes to promote chemosensitization are being recognized. These genes mediate the activation of several different chemotherapeutic agents, for example:

1. The *E. coli* DeoD gene that encodes for a purine nucleoside phosphorylase (PNP) converts 6-methylpurine-2'-deoxyribonucleoside (nontoxic) to 6-methylpurine (cytotoxic).
2. The *E. coli gpt* gene that encodes xanthine-guanine phosphoribosyl transferase (XGPRT, not found in mammalian cells) converts 6-thioguanine, following additional steps by enzymes normally found in human cells, to 6-thioguanosine monophosphate, an inhibitor of nucleic acid synthesis.
3. The human gene for a specific form of P-450 (designated 2B1, expressed in normal cells but not in tumor cells) heightens the sensitivity of tumor cells to cyclophosphamide (which is cytotoxic).

Immunomodulatory gene therapy

Immunomodulatory gene therapy is a method to induce cellular immune responses to metastatic lesions. The strategy involves injecting into the skin of a patient a suspension of irradiated tumor cells that have been transduced with a cytokine gene to stimulate a systemic immune response against tumor-specific antigens—in effect, vaccinating the patient against that specific cancer. The eventual success of immunotherapeutic strategies relies on whether tumor-specific antigens exist and on the extent to which a person's immune system is capable of recognizing these specific antigens and destroying tumor cells with which they are associated. Immunodulatory gene therapy is directed at enhancing both "arms" of these requirements.

Cytokines are factors involved in cell growth and development and they have effects on the immune system that could be put to therapeutic use. The basic idea of immunotherapy is to:

- modify tumor cells ex vivo (outside the body) with a cytokine gene, followed by
- transplanting the cytokine-gene modified cells back into the patient (after the cell has been irradiated to prevent further cell division), and
- letting the host's system create a systemic antitumor immune response.

Nitric oxide-donor nucleoside prodrugs as posible anticancer and antiviral agents

Chemistry of nitric oxide

The electrons that revolve around the nucleus of an atom occupy regions known as orbitals, each of which can be occupied by two electrons. An atom is most stable when each orbital is occupied by two electrons. An atom containing a single electron in its

outermost orbital is known as a free radical, as are molecules containing such atoms. Most free radicals react rapidly with other atoms, thereby filling the unpaired orbital; thus free radicals normally exist for only brief periods of time before combining with other atoms. Free radicals are diagramed with a dot next to the atomic symbol. Examples of biologically important free radicals are nitric oxide, •NO; superoxide anion, $O_2^{\bullet -}$; and hydroxyl radical, •OH. A number of free radicals such as •NO play important roles in the normal and abnormal functions within the body. Nitric oxide (nitrogen monoxide), written as •NO or simply NO, is a highly diffusible, stable and colorless gas composed of one atom each of nitrogen and oxygen. NO has a solubility of 2-3 mM in water and may undergo chemical reactions with a variety of atoms and radicals (Vander *et al.*, 2001).

Biosynthesis and inactivation of nitric oxide

The biosynthesis of NO is accomplished by oxidation of a terminal guanidine nitrogen of L-arginine, yielding L-citrulline as a co-product, and is catalyzed by a family of enzymes that are collectively called NO synthases (NOS) (Griffith & Stuehr, 1995). Three distinct isoforms of NOS have been identified in mammalian tissues, referred to as endothelial NOS (*e*NOS), neuronal NOS (*n*NOS), and inducible NOS (*i*NOS) (Venema *et al.*, 1997). There is an overall homology between the NO synthases and the cytochrome P450 reductase, but the latter enzyme lacks the binding site for L-arginine (Moncada, 1999). The chemistry of five electron oxidation of L-arginine by NOS to form NO and L-citrulline involves a series of co-factors including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4), molecular oxygen (O_2) and calmodulin (Griffith & Stuehr, 1995). Molecular oxygen is

introduced in the formation of N-hydroxy L-arginine, which is an intermediate in the synthesis and later on in the formation of L-citrulline (Moncada, 1999). Formation of NO from L-arginine and several NO donors is shown in Figure 22.

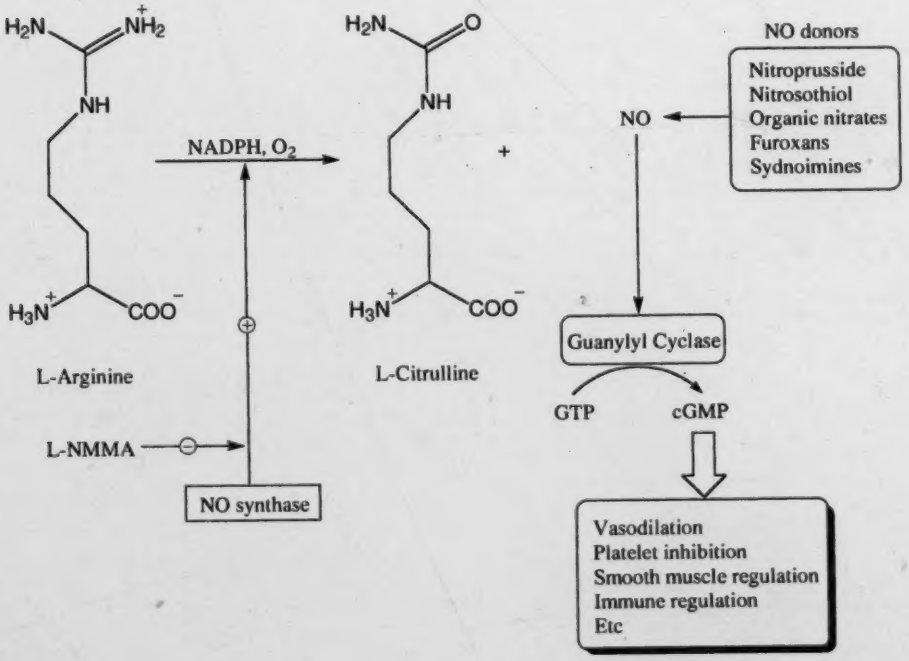


Figure 22. NO generation from L-arginine and NO donors and the formation of cGMP. L-NMMA inhibits NOS. Some NO donors such as furoxans and organic nitrates and nitrites require a thiol cofactor such as cysteine or glutathione to form NO (Katzung, 2001).

Activation of NOS by the influx of extracellular calcium, as in the case of the constitutive enzyme, or following the activation of the inducible NOS by lipopolysaccharide (LPS) or certain cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) results in the metabolism of L-

arginine to NO and L-citrulline (Bhagat & Vallance, 1996). The conversion of L-arginine to NO and L-citrulline is inhibited by several L-argenine competitors such as N^{ω}-monomethyl-L-arginine (L-NMMA), N^{ω}-nitro-L-arginine methylester (L-NAME) and aminoguanidine (AG). Some NO donors, e.g., oxygenated nitroprusside, spontaneously generate NO in aqueous solutions whereas others, such as furoxans and organic nitrates and nitrites, such as glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN), require the presence of a thiol compound such as cysteine (Cys) or glutathione (GSH) (Fukuyama *et. al.*, 1995). Once generated, NO interacts with the heme moiety of the soluble guanylyl cyclase, a magnesium-sensitive protein that exists as a heterodimer containing two heme molecules (Figure 22). This results in allosteric transformation and activation of the enzyme and leads to the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). Under basal conditions, guanylyl cyclase has no significant activity, but NO binds to heme with a high affinity and this binding initiates a conformational change in the ferrous heme complex to cause a greater than 400-fold activation of the enzyme. Interestingly, carbon monoxide, another endogenously synthesized gas proposed as a biological mediator working through cGMP, causes only a 4.4-fold activation of enzyme (Bhagat & Vallance, 1996). Activation of the soluble guanylyl cyclase by NO can be inhibited by methylene blue. The affinity of NO for iron is also responsible for its inhibitory effect on several enzymes, such as cytochrome P450, by interacting with the iron-sulfur centers of these enzymes. Until recently it had been accepted that NO is generated in tissues solely by metabolism of L-arginine. New evidence, however, indicates that NO is also generated nonenzymatically by direct

disproportion or reduction of nitrate ion to NO. This occurs under acidic and highly reducing conditions, e.g., in ischemia (Katzung, 2001).

Nitric oxide is inactivated by heme and by the free radical, superoxide. Thus, scavengers of superoxide anion such as superoxide dismutase (SOD) may protect NO, enhancing its potency and prolonging its duration of action. Conversely, interaction of nitric oxide with superoxide may generate the potent tissue-damaging moiety, peroxynitrite ($ONOO^-$), which has a high affinity for sulfhydryl groups and thus inactivates several key sulfhydryl-bearing enzymes. This effect of peroxynitrite is regulated by the cellular content of glutathione. Since glutathione is the major intracellular soluble sulfhydryl-containing compound, factors that regulate the biosynthesis and decomposition of glutathione may have important consequences. Glutathione also interacts with NO under physiologic conditions to generate *S*-Nitrosoglutathione, a more stable form of NO. Nitrosoglutathione may serve as an endogenous long-lived adduct or carrier of NO (Czapski & Goldstein, 1995).

Role of nitric oxide in the immune system

Inducible NO synthase, iNOS, was originally identified in macrophages (Moncada & Higgs, 1990). Circulating macrophages that are not activated do not produce NO, but if they are activated by certain cytokines or by lipopolysaccharide (LPS) there is *de novo* induction of NO synthase, which generates NO in large quantities and for very long periods. This differs from eNOS and nNOS, which release small quantities of NO in response to receptor stimulation. This iNOS in inflammatory cells uses NO as a cytostatic and cytotoxic agent (Nathan & Hibbs, 1991; Nussler & Billiar, 1993). NO inhibits

enzymes in the mitochondria, specially complex I and complex IV, as well as enzymes in the nucleus such as ribonucleotide reductase, responsible for synthesis of DNA. NO released by iNOS from murine macrophages is cytostatic and cytotoxic for protozoan parasites, fungal cells and bacteria. It has been shown that human macrophages can kill *Leishmania major* by the production of NO. Thus, NO either on its own, or in combination with oxygen radicals, might be a more important cytotoxic or cytostatic agent than oxygen radicals themselves (Moncada, 1999). Therefore, one of the most interesting aspects of the biology of NO is the way in which it changes from being a physiological mediator to a cytostatic/cytotoxic agent. One possible mechanism is that NO interacts with superoxide, which is also generated in inflammatory conditions, to form peroxynitrite. This compound is a highly oxidant species that will produce tissue damage. NO selectively and at physiological concentrations reversibly inhibits the last enzyme in the respiratory cycle, cytochrome *c* oxidase (complex IV). In this way NO plays an important role as a regulator of cell respiration. If, however, NO is produced in large quantities for long periods it will inhibit cytochrome *c* oxidase in a way in which oxygen will not be able to displace it from the enzyme. In such a situation, superoxide may be generated in the mitochondria itself and interact with NO to form peroxynitrite, which will block complex I and complex III irreversibly. So from inhibition of complex IV, which might be a physiological way in which NO can regulate respiration, NO could have adverse effects through the irreversible inhibition of complex I (Clementi *et al.*, 1998).

Nitric oxide as an anticancer and antiviral agent

Since the discovery that NO is biosynthesized in mammalian cells, NO has become a molecule of immense interest in medical research. NO production in response to immune activation, or inflammatory reaction, has been shown to serve as part of the host defense system against cancerous cells, and intracellular microbes and parasites (Kerwin *et al.*, 1995). In this regard activated macrophages that produce NO have been shown to inhibit DNA synthesis in tumor cells (Lepoivre *et al.*, 1991). Pretreatment of various cell lines with a NO-donor led to inhibition of the replication of poliovirus, picornavirus and rhinovirus (Reiss & Komatsu, 1998). In addition, IFN-γ treated cells produce NO that inhibits HSV-1 (herpesvirus) (Reiss & Komatsu, 1998). Other studies indicate that the antitumor activity of IL-1α may be mediated by the production of NO from tumor-derived endothelial cells (Chang *et al.*, 1996), and that macrophage NO synthesis delays progression of ultraviolet light-induced murine skin cancer through to an antitumor immune response (Yim *et al.*, 1993).

NO plays an important role in the action of a number of anticancer drugs due to its ability to influence various aspects of tumor biology including modulation of cell growth (Maragos *et al.*, 1993), apoptosis (Kitajima *et al.*, 1994), differentiation (Magrinat *et al.*, 1992), metastatic capability (Dong *et al.*, 1994), and tumor-induced immunosuppression (DiNapoli *et al.*, 1996). Thus, adriamycin stimulates NO production in EMT-6 cells, and *in vivo* adriamycin inhibits tumorigenesis partially via a NO-dependent, non-apoptotic mechanism (Lind *et al.*, 1997). Several antiviral acyclic nucleotide analogs activate expression of cytokine genes such as TNF-γ and IL-10 in macrophages, and IFN- γ in splenocytes, resulting in an enhanced production of NO

(Zidek *et al.*, 1999). A group of glyco-*S*-nitrosothiols was found to be cytotoxic to DU-145 human prostate cancer cells *in vitro* (Hou *et al.*, 1999). Induction of NO production by cytokines indicated that high levels of NO may contribute to the induction of apoptosis and inhibition of pancreatic tumor growth (Hajri *et al.*, 1998). A correlation exists between the intracellular levels of NO with the cytotoxicity of hydroxyguanidine in HL60 cells, where a higher toxicity was observed in hypoxic cells relative to oxic cells (Everett *et al.*, 1996). The hypoxic cell radiosensitization effects of the NO-donor $Et_2N[N(NO)]^- Na^+$, coupled with the vasodilatory effect of NO on tumor vasculature, suggest that an agent of this type opens a new avenue in radiation oncology treatment (Mitchell *et al.*, 1993). The rate and extent of NO release from NO/nucleophile adducts, which release NO spontaneously in solution, correlated with inhibition of DNA synthesis in A375 human melanoma cells (Maragos *et al.*, 1993). Tamoxifen has been shown to induce iNOS, producing NO that is cytotoxic (Loo *et al.*, 1998). NO-induced apoptosis in macrophages activated with cisplatin and IFN-γ requires activation of an endonuclease (Ranjan *et al.*, 1998). NO-donor agents have been shown to enhance melphalan cytoxicity in breast cancer MCF-7 cells, possibly by delaying entry into the S-phase and a G2/M block (Cook *et al.*, 1997). Using a number of aromatic *N*-nitroso compounds, it was shown that the extent of NO generation was the reciprocal of ID_{50} growth inhibition (cytotoxicity) (Tanno *et al.*, 1997). These data collectively indicate that NO exhibits a cytotoxic effect and/or enhances the cytotoxic effect of anticancer drugs. However, there are a few instances where NO is detrimental to anticancer activity. For example, NO generated from a NO-donor drug was shown to prevent taxol-induced apoptosis, and this was attributed to inhibition of an IL-1β converting enzyme-like protease cascade (human

neuroblastoma cell line, NB-39-nu) (Ogura *et al.*, 1998). A related study showed that the protective effect of NO on taxol-induced apoptosis may be partially caused by inhibiting entrance of the cells into the G2/M phase (human leukemia HL-60 cells) (Pae *et al.*, 1999). These data indicate that the efficacy of an NO-donor anticancer drug may be dependent upon the specific cell line. Recently, several mechanisms have been proposed to account for anticancer and antiviral effects of NO. NO is known to degrade certain iron-containing prosthetic groups resulting in inhibition of the mitochondrial respiratory chain, DNA synthesis and aconitase activity. Other proteins containing iron-sulfur clusters may be influenced as well, and NO has been reported to increase iron loss from cells as well as the release of iron from ferritin. NO can also react with superoxide produced from activated macrophages to form peroxynitrite in which the protonated form (HOONO) acts as a potent chemical oxidant, resulting in modification of protein functions and causing DNA damage (Hibbs *et al.*, 1988).

Current major classes of nitric oxide donors

Due to the instability and inconvenient handling of aqueous solutions of authentic NO, there is an increasing interest in using compounds capable of generating NO in situ that are classified as NO donors. All nitrogen-oxygen bonded compounds have the potential to decompose, be oxidized, or be reduced to produce reactive nitrogen species. Major classes of current NO donors along with their individual pathway of NO generation are summarized in Table 2.

Table 2. Current major classes of NO donors (Wang *et al.*, 2002).

Name	Pathway of NO Generation	
	Non-enzymatic	Enzymatic
Organic nitrates	thiols	Cyt-P450, GST and a membrane-bound enzyme
Organic nitrites	hydrolysis and trans-nitrosation; thiols; light; heat	cytosolic and microsomal enzymes; xanthine oxidase
Metal-NO complexes	light; thiols; reductants; nucleophiles	a membrane-bound enzyme
N-Nitrosamines	OH; light	Cyt-P450 related enzymes
N-Hydroxyl nitrosamines	light; heat	peroxidases
Nitrosamines	thiols; light	?
Nitrosothiols	spontaneous; enhanced by thiols, light and metal ions	unknown enzymes
C-nitroso compounds	light; heat	?
Diazetine dioxides	spontaneous; thiols	?
Furoxans and benzofuroxans	thiols	unknown enzymes
Oxatriazole-5-imines	thiols	?
Sydnonimines	spontaneous, enhanced by light, oxidants and pH > 5	prodrugs require enzymatic hydrolysis
Oximes	spontaneous; O_2/Fe^{III}-porphyrin	?
Hydroxylamines	Autoxidation, enhanced by metal ions	catalase/H_2O_2
N-Hydroxyguanidines	oxidants	NOSs, Cyt-P450
Hydroxyureas	H_2O_2/CuZn-SOD or ceruloplasimin; H_2O_2/Cu^{2+}; heme-containing proteins	peroxydase
Diazeniumdiolates	Spontaneous, enhanced by acid	?

The structural dissimilarities of the diverse NO donors have led to remarkably varied chemical reactivities and NO-release kinetics. A number of studies have shown NO generation can be induced by thiols, based on either reduction or transnitrosation. In fact, thiols represent a primary cofactor required for NO release from furoxans, organic nitrite, nitrate, and other nitro compounds. Many NO donors are so heat/light sensitive that they can decompose spontaneously if precautionary measures are not taken. Some compounds are very hygroscopic and NO release may be pH-dependent. Ligands can even induce and facilitate NO release from some metal-NO complexes. On the contrary, some NO donors are relatively stable and do not generate NO without the presence of oxidants or metal-ion catalysis (Wang *et al.*, 2002).

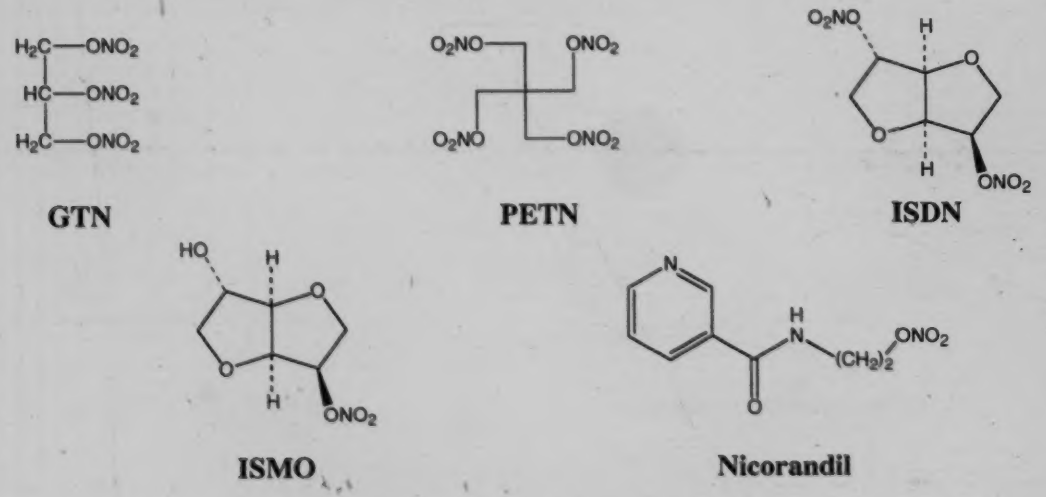


Figure 23. Representative organic nitrates.

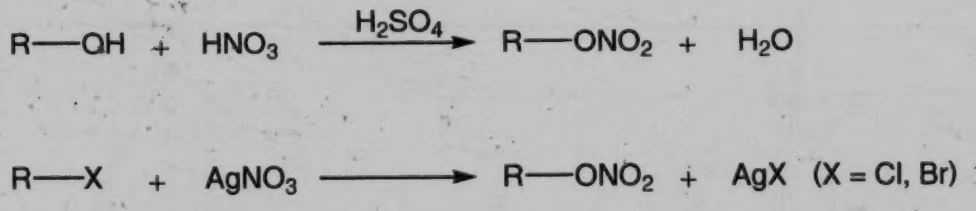
Organic nitrates are the most well-known class of NO donors

Organic nitrates ($RONO_2$) are nitric acid esters of mono- and polyhydric alcohols, representing the oldest class of NO donors that have been clinically applied. Representative organic nitrates include glyceryl trinitrate (GTN), pentaerythrityl

tetranitrate (PETN), isosorbide dinitrate (ISDN), isosorbide 5-mononitrate (ISMO), and nicorandil (Figure 23).

Synthesis

In general, organic nitrates can be readily prepared by esterification of the corresponding alcohols using nitric acid and sulfuric acid, or the treatment of reactive alkyl halides with $AgNO_3$ (Baker & Heggs, 1954) (Scheme 4).



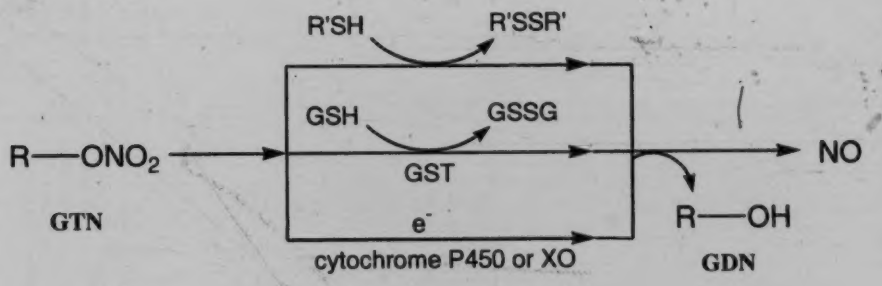
Scheme 4. General methods for synthesis of organic nitrates.

Physical properties

Most organic nitrates are only sparingly soluble in water. When properly sealed and protected from light, their solution can be stored in ethanol or dimethyl sulfoxide (DMSO) for months to years. Organic nitrates are generally stable in neutral or weakly acidic aqueous solution (Honeyman & Morgan, 1957). Under strong alkaline conditions, they are susceptible to hydrolysis (S_N2 nucleophilic substitution to give alcohol and nitrate), β-H elimination (forming alkene), and α-H elimination (producing aldehyde and nitrite) (Capellos *et al.*, 1984; Baker & Easty, 1952).

Metabolism and bioconversion

The major biological effects of nitrates are attributable to the formation of NO. NO release from organic nitrates requires either enzymatic or nonenzymatic bioactivation where a three-electron reduction is involved (Torfgard & Ahlner, 1994; Bennett *et al.*, 1989). Although the biochemical process of NO release from GTN has not been fully defined, it is likely that multiple intracellular and extracellular pathways (Scheme 5) contribute to NO formation from these compounds *in vivo* (Ahlner *et al.*, 1991; Bennett *et al.*, 1994).



Scheme 5. Enzymatic and nonenzymatic metabolism pathways for GTN.

When GTN is absorbed, it is rapidly denitrated into water-soluble 1,2-glyceryl dinitrate (GDN), 1,3-glyceryl dinitrate, 1- and 2-mononitrates (GMN), and glycerol by more than one enzyme system. Servent *et al.* (1989) studied GTN denitration in rat liver tissue where the main metabolic transformation of GTN by liver microsoms is a cytochrome P-450-dependent reduction by NADPH leading to di- and mono-nitrates, GDN and GMN, and NO which is able to bind to cytochrome P-450 (Reaction 1).

$$RO\text{-}NO_2 + \text{P-450-Fe(II)} \xrightarrow{NADPH} ROH + \text{P-450-Fe(II)-NO} \qquad (1)$$

Kurz *et al.* (1993) studied GTN metabolism in dog carotid arteries. Though there is no doubt that GSH-S-transferases (GSTs) are involved in converting GTN to GDN and NO_2^-, GSTs are not involved in the bioactivation of GTN. The denitration reactions are shown in reaction (2) and (3) (Kurz *et al.*, 1993).

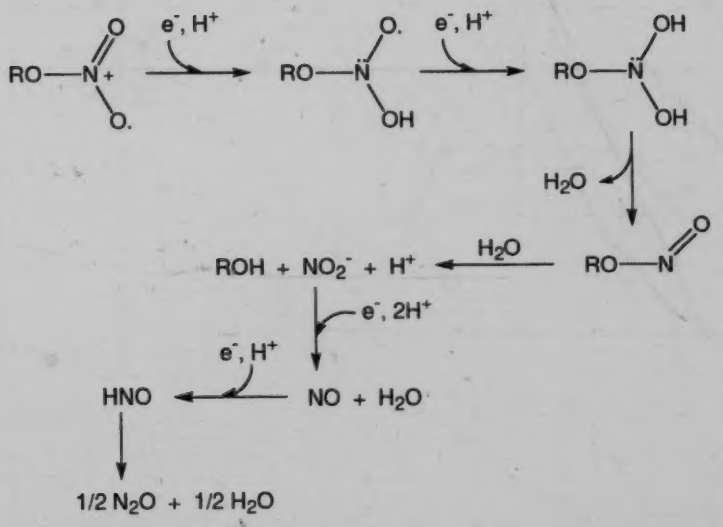
$$RO\text{-}NO_2 + 2GSH \xrightarrow{\text{GSH-S-transferase}} GSSG + ROH + NO_2^- \quad (2)$$

$$H^+ + GSSG + NADPH \xrightarrow{\text{GSH-reductase}} 2GSH + NADP^+ \quad (3)$$

MaCurie *et al.* (1994) reported that GTN bioactivation could be mediated by a flavin protein. Wong & Fukuto (1999) further examined the intimate chemistry among flavin, thiol, and GTN. In their model, they found that GTN could undergoes a two electron reduction to form the corresponding nitrite ester in the presence of flavin protein and NADPH (Reaction 4).

$$RO\text{-}NO_2 + 2e^- + 2H^+ \longrightarrow RO\text{-}NO + H_2O \quad (4)$$

Hydrolysis of nitrite ester generates NO_2^-, which is then further subjected to 2-electron reduction to HNO. Dimerization of HNO can produce N_2O, which can easily be detected in the experiments (Scheme 6) (Wong & Fukuto, 1999).



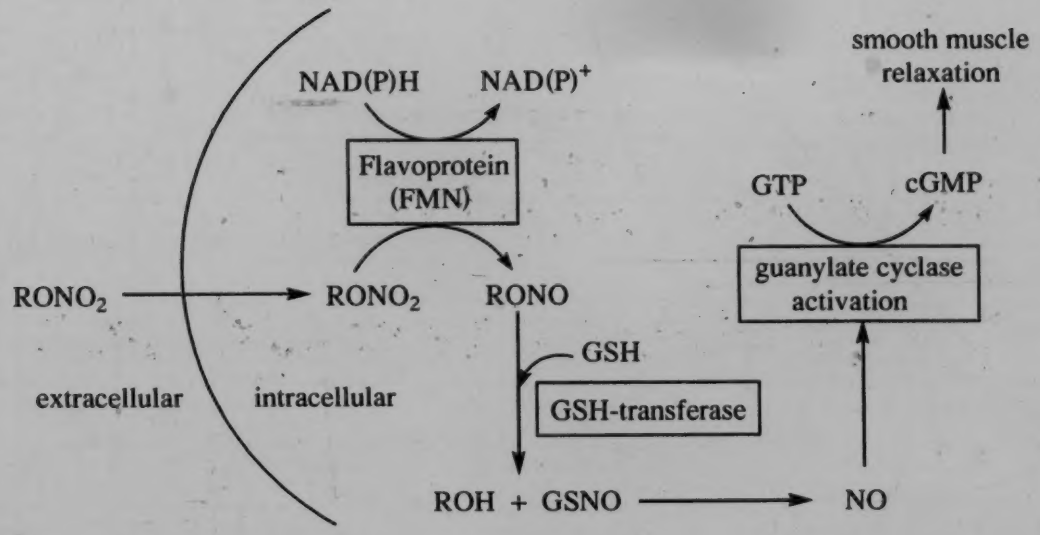
Scheme 6. Reaction of organic nitrate esters (Wong & Fukuto, 1999).

Nitrate esters can also react with GSH to form GSNO; this reaction is probably catalyzed by GST. GSNO reacting with flavin mononucleotide (FMN)/NADH can release NO (Reaction 5, 6). The presence of FMN can increase NO release dramatically (Wong & Fukuto, 1999).

$$GSNO + NADH \longrightarrow NAD^+ + GS^- + HNO \quad (5)$$

$$GSNO + HNO \longrightarrow GSN(OH)NO \longrightarrow GSH + 2NO \quad (6)$$

In summary, a possible mechanism for bioactivation of organic nitrate esters is presented in Scheme 7. First, organic nitrate esters are taken up by cells; then a flavin protein (a possible membrane-bound protein) reduces it to an organic nitrite ester; nitrite ester further reacts with endogenous GSH to form GSNO in the presence of GST; GSNO will eventually release NO by various mechanisms (Wong & Fukuto, 1999).



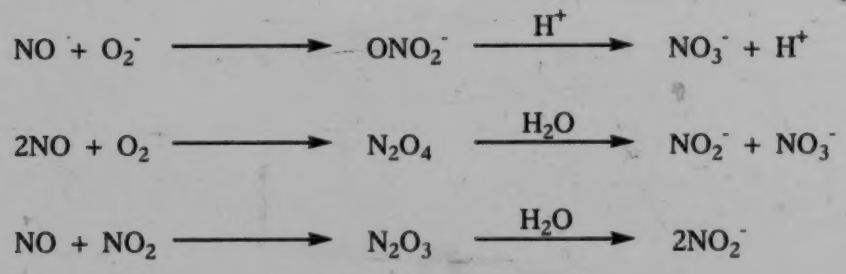
Scheme 7. Possible reaction pathway for $RONO_2$ reduction to NO (Wong & Fukuto, 1999).

Nitric oxide release determination

The measurement of NO *per se* is rather difficult since NO is a molecule with a short half-life, and it reacts rapidly with free oxygen, oxygen radicals, redox metals, sulfhydryls, disulfides, and oxygenated hemoglobin (Stamler *et al.*, 1992). NO as a free radical may be measured by a rather complex electron paramagnetic resonance (EPR) method with nitroso or hemoglobin spin traps (Arroyo & Kohno, 1991; Wannmalm *et al.*, 1990). The local cellular output (but not NO concentration) may be monitored by the porphyrin electrode described by Vallance *et al.* (1995). Other methods are based on spectrophotometric measurement of the conversion of oxyhemoglobin to methemoglobin in the presence of NO (Kelm *et al.*, 1988), chemiluminescence detection (Zafiriou & McFarland, 1980) and by fluorescence detection (Misco *et al.*, 1993). All of the methods mentioned above can be applied to *ex vivo* or *in vitro* studies. However, NO release can

be determined spectrophotometrically by measuring the accumulation of its stable degradation products, nitrite (NO_2^-) and nitrate (NO_3^-), according to the Griess reaction (Griess, 1879) supplemented with the reduction of nitrate to nitrite by NADPH-dependent reductase or with cadmium reduction (Schmidt *et al.*, 1992). The ratio of nitrite/nitrate is variable, depending on the pathway of oxidative inactivation (Scheme 8): reaction with superoxide anions will predominantly yield nitrate; with molecular oxygen, nitrite (Schmidt *et al.*, 1992).

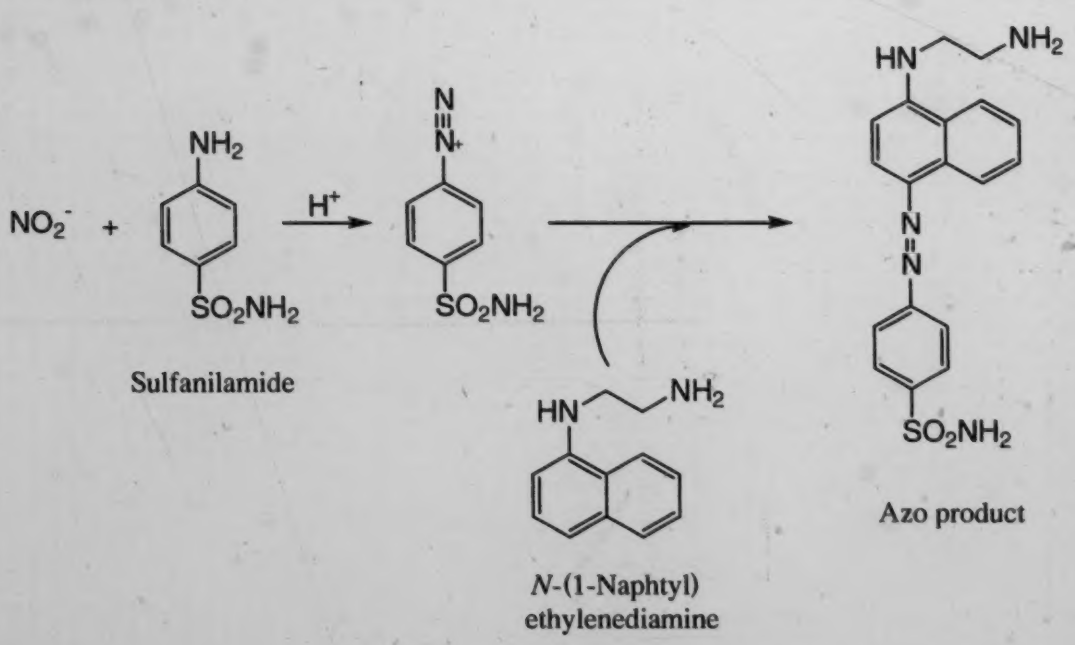
The Griess reaction is based on a simple colorimetric reaction between nitrite, sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) under acidic conditions (Scheme 9) to produce a pink/magenta azo product with a maximum absorbance at 543 nm.



Scheme 8. Formation of two stable breakdown products, nitrate and nitrite, from oxidative inactivation of NO.

Sulfanilamide and NED compete for nitrite in the Griess reaction, thus greater sensitivity is achieved when the two components are added sequentially. To ensure accurate nitrite quantitation, a reference curve with a nitrite standard, with the same buffer used for experimental samples is prepared. Due to substances that interfere with

the Griess reaction, different levels of sensitivity may be achieved in different buffers (Giovannoni *et al.*, 1997).

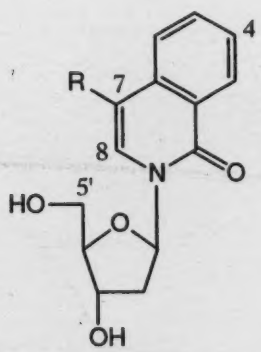


Scheme 9. Chemical reactions involved in the measurement of using the Griess reagents.

2. OBJECTIVES

1. Design, synthesis and biological evaluation of unnatural 7-substituted isocarbostyril nucleosides for use as antiviral/anticancer/gene therapy agents

The objectives of this study involve the design, synthesis and biological evaluation (*in vitro* and *in vivo*) of 5-substituted-2'-deoxyuridine mimics, having an unnatural 7-substituted-isocarbostyril (bicyclic 2-pyridone) moiety (Figure 24) in place of the natural thymine base, as potential antiviral and/or anticancer agents to treat and/or image HSV-1 thymidine kinase positive (TK^+) gene transfected tumors (gene therapy of cancer).



1, R = —C≡C–CH_3
7, R = H
9, 4,7-dinitro
11, R = I
13, R = CF_3
15, R = CN
18, R = (*E*)-CH=CH-I
20, R = —C≡C–H
23, R = —C≡C–I
24, R = —C≡C–Br

Figure 24. Structures of unnatural 7-substituted isocarbostyril nucleosides.

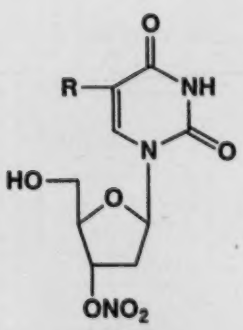
These novel unnatural 7-substituted-1-(β-D-2'-deoxyribofuranosyl) isocarbostyryls **1**, **7**, **9**, **11**, **13**, **15**, **18**, **20**, **23** and **24** could i) act as irreversible inhibitors of thymidylate synthase (TS) upon reaction of the –SH group of TS at the electron deficient C-8 position of the isocarbostyril ring to exhibit a cytotoxic effect (5'-monophosphates), ii) stop DNA chain elongation after incorporation to form "bogus

DNA" resulting in a cytotoxic effect (5'-triphosphates), iii) are much more lipophilic than natural pyrimidine nucleosides allowing passage across the blood-brain-barrier (BBB) to treat brain viral infections and tumors, iv) constitute a potentially new class of 3rd generation antiviral (-C≡C-, -I, $-CF_3$, *E*-CH=CH-I, -C≡C-I) or anticancer (I, F), and as antiviral agents to image and treat HSV-1 TK^+ gene transfected tumors (gene therapy of cancer) that v) may extend the genetic alphabet beyond the natural A-T and C-G pairs.

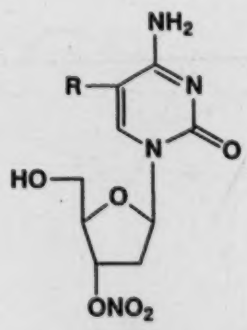
2. Design, synthesis and biological evaluation of 3'- and 5'-nitrooxy pyrimidine nucleosides as NO-donor/nucleoside hybrid drugs for evaluation as anticancer and antiviral agents

The significant therapeutic potential associated with the rational control of nitric oxide (NO) release has stimulated intense pharmaceutical interest, since the biological action of NO relates directly to the specific tissue or cell type in which it is released. In this regard, NO mediates some of the immune system's antiviral and antitumor effects, radiosensitizes (increases killing of cancer cells by radioactive emissions) hypoxic (oxygen deficient) tumor cells, and arrests the growth of some tumor cells in culture. A class of compounds called 5-substituted-2'-deoxyuridines is known to exhibit antiviral and/or anticancer activities. Their primary mechanism of action is attributed to the inhibition of certain enzymes such as DNA polymerase and thymidylate synthetase (TS) that are involved in the synthesis of DNA by rapidly growing tumors in which there is rapid cell multiplication compared to normal tissue. It is proposed that a novel class of 5-substituted-3'- and 5'-nitrooxy pyrimidine nucleosides (Figure 25) could act as "double-barreled anticancer agents" due to their dual ability to release NO inside

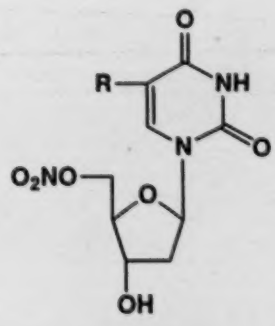
the tumor thereby killing the cancer cells, and by the termination of DNA synthesis that retards or reduces tumor cell multiplication. Therefore, the goals of this research involve the design, synthesis, and biological evaluation (*in vitro* and *in vivo*) of 5-substituted-2'-deoxyuridine having a 3'- and 5'-nitrooxy (-ONO_2) substituent (in place of 3'- and 5'-OH in natural nucleoside compounds) in the sugar ring (**35a-d** and **40a-c**) and 5-substituted-2'-deoxycytidine having a 3'-nitrooxy substituent (**38a-c**) to simultaneously release nitric oxide (NO) and prevent tumor growth and viral replication.



35a, R = H
35b, R = Me
35c, R = F
35d, R = I



38a, R = H
38b, R = Me
38c, R = I



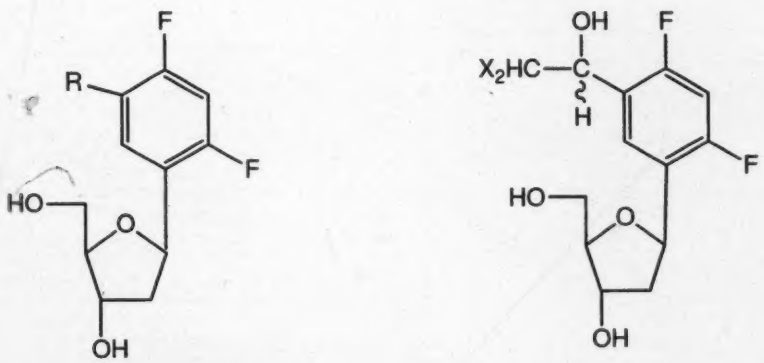
40a, R = H
40b, R = Me
40c, R = I

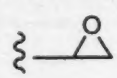
Figure 25. Structures of unnatural 5-substituted 3'- and 5'-nitrooxy pyrimidine nucleosides.

3. Design, synthesis and biological evaluation of unnatural 5-substituted-2,4-difluorobenzene *C*-nucleosides for use as antiviral/anticancer/gene therapy agents

The objectives of this research involve the design, synthesis and biological evaluation (*in vitro* and *in vivo*) of 5-substituted-2,4-difluoro-1-(β-D-2'-

deoxyribofuranosyl)]benzenes **45-48** and **51-52** (Figure 26) as 2'-deoxyuridine mimics, having an unnatural 5-substituted-2,4-difluorobenzene moiety in place of the natural thymine base, as potential antiviral and/or anticancer agents to treat and/or image HSV-1 thymidine kinase positive (TK^+) gene transfected tumors (gene therapy of cancer).



45, R = -$CH(OH)CH_2Br$
46, R = -$CH(OH)CH_2I$
47, R = -$CH(OCH_3)CH_2I$
48, R = 

51, X = Br
52, X = I

Figure 26. Structures of unnatural 5-substituted-2,4-difluorobenzene C-nucleosides as 5-substituted-2'-deoxy pyrimidine nucleoside mimics.

These novel compounds are isosteric mimics (isoelectronic, steric mimic) with natural thymidine, therefore it is expected that these unnatural analogs of thymidine will be incorporated into DNA (like thymidine) to produce bogus DNA resulting in an antiviral and/or anticancer effect. Furthermore, it is predicted that these unnatural mimics will be stable to glycosidic bond cleavage by pyrimidine phosphorylases (unlike thymidine) since they are *C*-nucleosides rather than the natural *N*-nucleosides such as thymidine. In view of the fact that these unnatural analogs are very lipophilic, it is

expected that unlike natural nucleosides, which are very hydrophilic, they will have the ability to enter brain. This valuable property allows their potential use in treating brain viral infections and treating brain tumors.

3. RESULTS AND DISCUSSION

1. Synthesis of unnatural 7-substituted isocarbostyril nucleosides as possible antiviral/anticancer agents

The specific *H*-bonding patterns of the Watson-Crick A-T and G-C base pairs serve an important role with respect to the storage and replication of biological information (Komberg & Baker, 1992). Accordingly, the design of enzymatically replicable "unnatural base pairs" may constitute a method to expand the biological and chemical genetic alphabet potential of DNA beyond the natural A-T and G-C pairs. McMinn *et al.* (1999) reported the unnatural hydrophobic 7-propynylisocarbostyril nucleoside mimic **1** that contains an isocarbostyril moiety, in place of the natural uracil base, that can not form *H*-bonds (Figure 27).

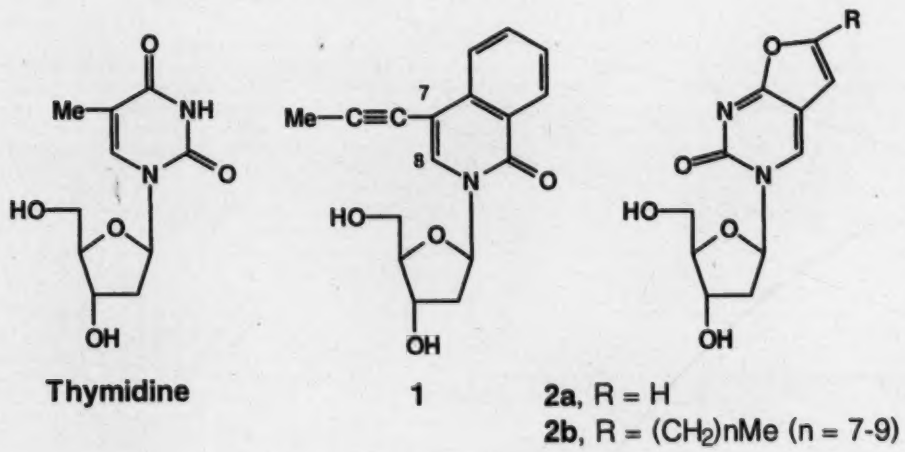


Figure 27. Structure of thymidine, 1-(2-deoxy-β-D-ribofuranosyl)-7-propynyl isocarbostyril (**1**), and some unusual 3-(2-deoxy-β-D-ribofuranosyl)-6-substituted-2,3-dihydrofuro[2,3-d]pyrimido-2-one derivatives **2**.

The unnatural self-pair **1-1** is able to significantly stabilize duplex DNA relative to the natural A-T and G-C base pairs. In spite of the fact that the mimic **1** does not have shape complimentarity to any native base, the 5'-triphosphate of **1** is still incorporated opposite itself (**1-1** pair) with reasonable efficiency by the Klenow fragment of *E. coli* DNA polymerase 1 (KF) such that the **1-1** base pair is the most "orthogonal" base pair relative to the natural bases, reported to date. Although KF (DNA polymerase) inserts **1**-triphosphate opposite **1** with reasonable efficiency, continued synthesis (chain elongation) proceeds inefficiently. This is attributed to the assumption that the unnatural base pair **1-1** assumes a geometry that incorrectly positions the 3'-OH of the growing strand for nucleophilic reaction with the incoming nucleoside triphosphate (McMinn *et al.*, 1999). These results suggest that nucleoside mimics such as **1** should be cytotoxic to rapidly multiplying cancer cells (inhibit tumor growth) and/or act as antiviral agents due to their selective phosphorylation by virus-infected cells (De Clercq & Walker, 1984). Credence for this latter concept is provided by the discovery that the unusual bicyclic 2,3-dihydrofuro[2,3-d]pyrimidin-2-one moiety present in compounds **2b**, which are derivatives of **2a** described previously (Kumar *et al.*, 1991; Kumar *et al.*, 1996) (Figure 27), exhibit potent and selective inhibition (300-fold greater potency than acyclovir with no detectable *in vitro* cytotoxicity) of varicella-zoster virus (VZV) (McGuigan *et al.*, 1999). This latter observation indicates that certain large bicyclic heterocyclic base moieties are tolerated with respect to retention of antiviral efficacy. It was therefore anticipated that 7-substituted nucleoside mimic derivatives of **1** may be useful as anticancer or antiviral agents, and as radiopharmaceutical agents to image (Morin *et al.*, 1997), or chemotherapeutic agents to treat (Oldfield *et al.*, 1993), herpes simplex virus

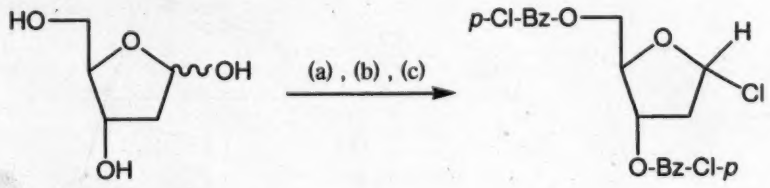
type-1 thymidine kinase positive (HSV-1 TK^+) gene-transfected tumors (gene therapy of cancer) (Wiebe *et al.*, 1997).

Modification of pyrimidine nucleoside bases can bestow new properties (oral bioavailability, metabolic stability, and pharmacokinetic) (Perigaud *et al.*, 1992), which the natural bases lack, and, when incorporated into nucleic acids by enzymatic processes, can further alter the structure and/or function of these polymers. In this respect, 2'-deoxyuridine derivatives, which possess a C-5 substituent two-carbon atoms in length, generally exhibit antiviral, but not anticancer, activity. Structure-activity relationship correlations (SARCs) for 5-olefinic 2'-deoxyuridine analogs showed that optimum inhibition of HSV-1 in vitro occurred when the 5-substituent was unsaturated and conjugated with the uracil ring, was not longer than four carbon atoms, had the (*E*)-stereochemistry, and included a hydrophobic electronegative functionality such as (*E*)-CH=CH-I (Goodchild *et al.*, 1983). There has been considerable interest in 5-alkynyl pyrimidine nucleosides to treat VZV infections. Although 5-ethynyl-2'-deoxyuridine (5-C≡CH) was highly active against HSV-1, CMV, and VZV in vitro, it was also cytotoxic. In contrast, 5-(-C≡C-CH_3) derivatives of 2'-deoxyuridine and arabinouridine (Zonavir) exhibited highly selective anti-VZV activity and they were noncytotoxic. Thus changing the C-5 substituent from -C≡CH to -C≡C-CH_3 greatly increased VZV selectivity and decreased cytotoxicity, effects attributed to an inability of the -C≡C-CH_3 analogs to act as substrates for cellular TK, or to decreased affinity of the monophosphate for thymidylate synthase (TS) (Rahim *et al.*, 1992). It was, therefore, anticipated that nucleoside mimic derivatives of **1**, possessing a C-7 two- or three-carbon substituent, may be cytotoxic to rapidly multiplying cancer cells (inhibit tumor growth) and/or act as antiviral agents due

to their selective phosphorylation by virus-infected cells, and as radiopharmaceutical agents to image, or chemotherapeutic agents to treat herpes simplex virus type-1 thymidine kinase positive (HSV-1 TK^+) gene-transfected tumors (gene therapy of cancer). The synthesis, and antiviral-anticancer activities for a group of 1-(2-deoxy-β-D-ribofuranosyl)-7-substituted-isocarbostyrils (**1**, **7**, **9**, **11**, **13**, **15**, **18**, **20**, **23**, **24**) designed as 5-substituted-2'-deoxyuridine (thymidine) mimics are reported herein.

Chemistry

Nucleoside mimics are frequently synthesized by reaction of an *O*-trimethylsilylated base mimic (McMinn *et al.*, 1999), or an arylcadmium reagent (Wang *et al.*, 2001), with the well known Hoffer α-chloro sugar synthon [3,5-bis-*O*-(*p*-toluoyl)-2-deoxy-α-D-ribofuranosyl chloride] (Hoffer, 1960). Accordingly, 3,5-bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-α-D-ribofuranosyl chloride (**3**) was used in place of the Hoffer α-chloro sugar synthon since it is a more stable and readily accessible crystalline reagent (Wang *et al.*, 2001) (Scheme 10).



2-deoxy-D-ribose **3**

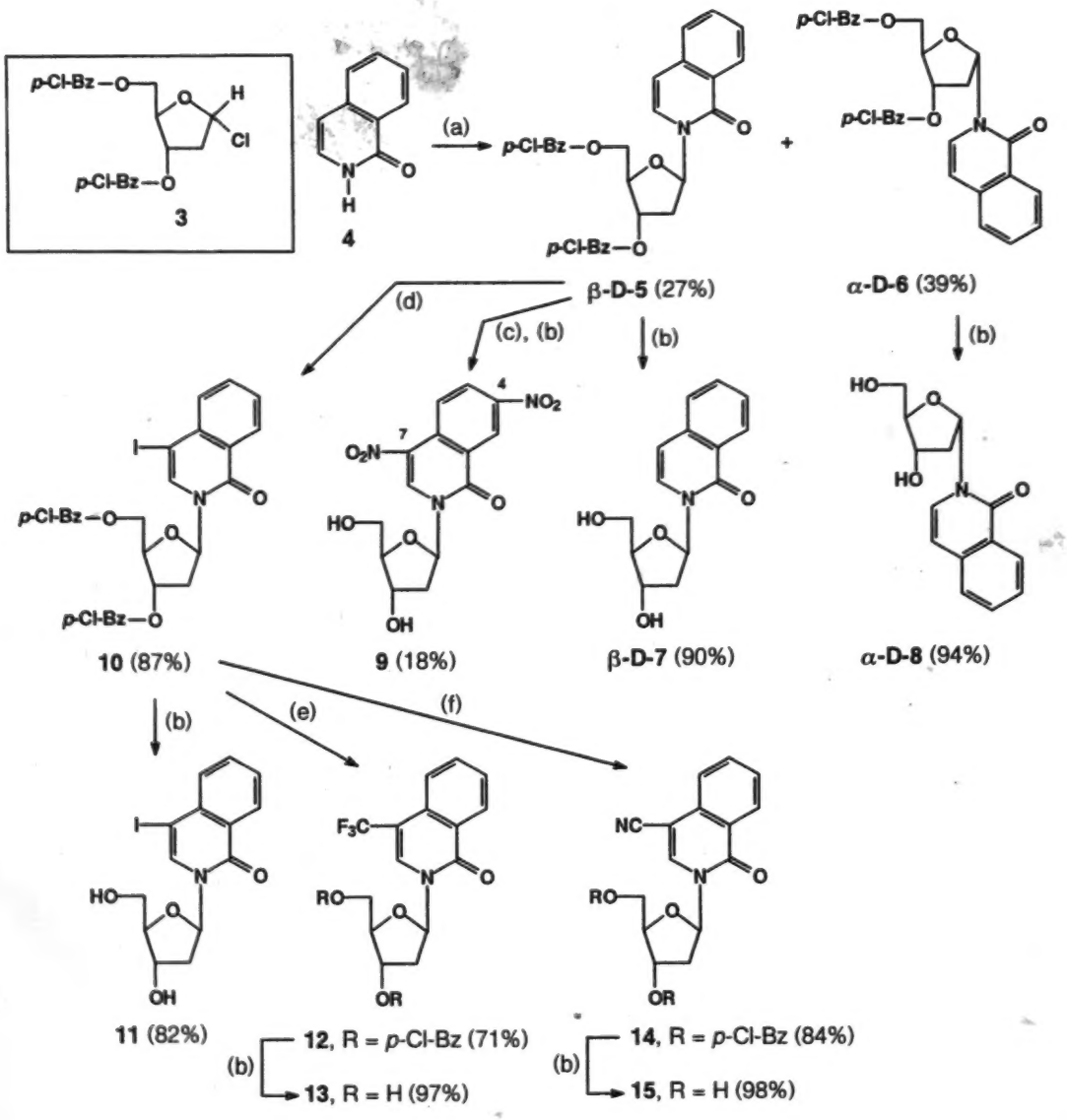
Scheme 10. Synthesis of 3,5-bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-α-D-ribofuranosyl chloride (**3**).

Reagents and conditions: (a) MeOH, acetyl chloride, 45 min at 25 °C and then pyridine; (b) pyridine, 4-dimethylaminopyridine, 4-chlorobenzoyl chloride, 1 h at 0 °C and then 12 h at 25 °C; (c) HOAc, dry HCl gas, 0 °C, 7-10 min.

The intermediates in this synthesis were not purified. Addition of a small amount of acetyl chloride to the saturated acetic acid-HCl solution before adding the protected ribose methyl ether maintains the reaction mixture in a dry state and affords higher product yield. The α-chloro sugar **3** prepared via this method is sufficiently pure for immediate use without further purification.

Coupling the α-chloro sugar **3** to the 2-*O*-trimethylsilyl derivative of isocarbostyril (**4**), which was generated from the reaction of **4** with *N,O*-bis(trimethylsilyl)acetamide, was performed in MeCN at 25 °C (Scheme 11). As reported for a similar reaction using 3,5-bis-*O*-(*p*-toluoyl)-2-deoxy-α-D-ribofuranosyl chloride (McMinn *et al.*, 1999), this coupling reaction yielded a mixture of the 3,5-bis-*O*-(*p*-chlorobenzoyl) β-D-**5** (27%) and α-D-**6** (39%) anomers that were readily separated by flash silica gel column chromatography. Subsequent removal of the *O*-(*p*-chlorobenzoyl) protecting groups by treatment with NaOMe afforded β-D-**7** (90%) and α-D-**8** (94%), respectively.

The configuration at the C-1' carbon, and the conformation, of the β-D-**7** and α-D-**8** anomers possessing an isocarbostyril moiety was analyzed by nuclear Overhauser enhancement (NOE) ^{1}H NMR difference spectroscopy (Figure 28). Selective irradiation of the H-4' signal (β-D-**7**) resulted in an enhancement of the H-1' signal (6.8%), and irradiation of the H-2" signal gave a 6.0% enhancement of the H-1' signal, which support the assignment of the β-configuration (Walker *et al.*, 1997). The C-8 hydrogen of the isocarbostyril moiety is oriented in the direction of the sugar ring since NOE enhancements of H-8 were observed upon selective irradiation of H-2' (6.4%) and H-3' (3.6%).



Scheme 11. Synthesis of 1-(2-deoxy-β-D-ribofuranosyl)-7-substituted-isocarbostyrils **7**, **9**, **11**, **13** and **15**.

Reagents and conditions: (a) *N*,*O*-bis(trimethylsilyl)acetamide, MeCN, 25 °C, 30 min, and then **3**, $SnCl_4$, 0 °C, 30 min; (b) NaOMe, MeOH, 25 °C, 30 min; (c) NH_4NO_3, $(CF_3CO)_2O$, 25 °C, 1 h; (d) ICl, CH_2Cl_2, reflux; (e) CuI, KF, DMF at 60 °C, and then $ClCF_2CO_2Me$, 120 °C, 6 h; (f) $Zn(CN)_2$, $(PPh_3)_4Pd$, DMF, 80-90 °C, 6 h.

In contrast, irradiation of the H-1' signal of α-D-**8** provided enhancements of *cis*-H-2' (6.4%) and H-3' (1.9%). The failure to observe an enhancement of H-1' upon irradiation of H-4' indicates these two hydrogens are on opposite faces of the sugar moiety, which is indicative of the α-configuration (Walker *et al.*, 1997).

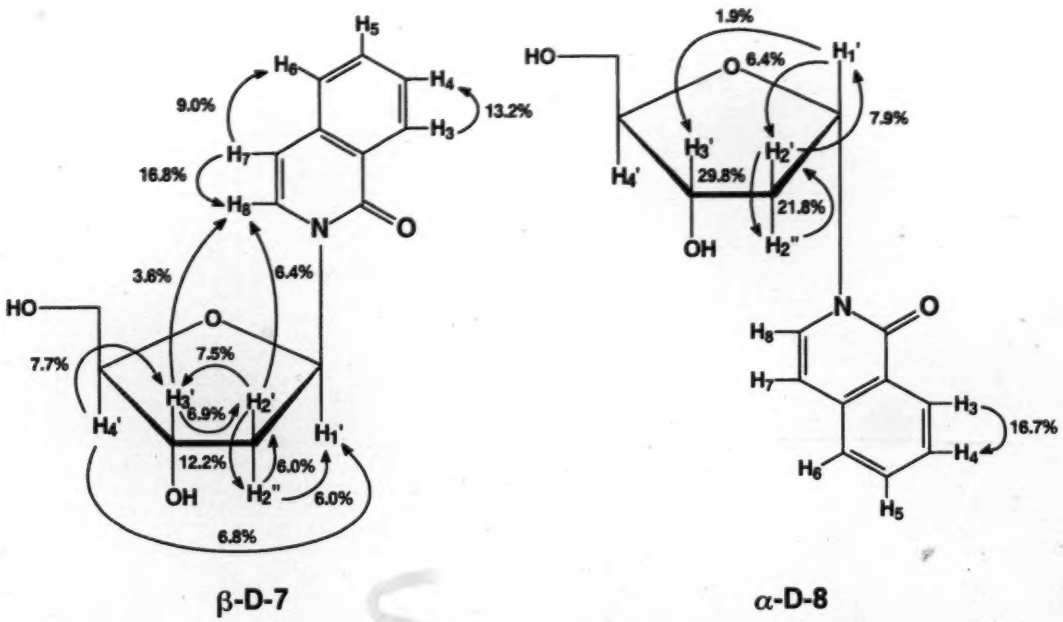


Figure 28. Some NOE studies to study the configuration and conformation of 1-(2-deoxy-β-D-ribofuranosyl)isocarbostyril (β-D-**7**), and 1-(2-deoxy-α-D-ribofuranosyl) isocarbostyril (α-D-**8**) in MeOH-d_4 at 22 °C.

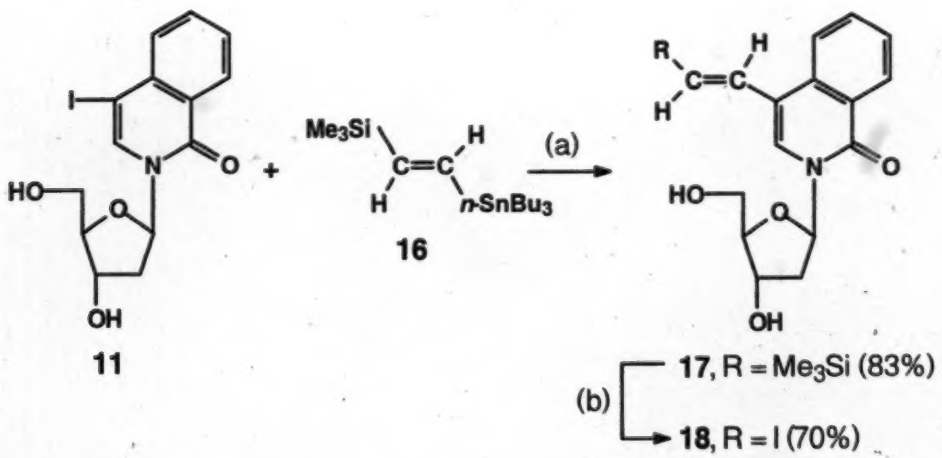
Nitration (Crivello, 1981) of the 3,5-bis-*O*-(*p*-chlorobenzoyl) ester β-D-**5** using ammonium nitrate in trifluoroacetic anhydride afforded the 4,7-dinitro derivative, which on deprotection with NaOMe in MeOH at 25 ° C, gave 1-(2-deoxy-β-D-ribofuranosyl)-4,7-dinitroisocarbostyril (**9**, 18% overall yield from β-D-**5**). The ^{1}H NMR spectrum for **9** indicated that nitration occurred at the expected C-7 position since H-8 appeared as a singlet at δ 9.57, and at either C-4 or C-5 since a doublet (J = 2.4 Hz), doublet (J = 9.1

Hz) and a doublet of doublets (J = 9.1, 2.4 Hz) were present at δ 8.93, 8.75 and 8.66, respectively, for three phenyl protons located on the isocarbostyril ring system. A semi-empirical PM3 calculation showed the electron densities in isocarbostyril (**4**) are C-3 (-0.031), C-4 (-0.129), C-5 (-0.065), C-6 (-0.117) and C-7 (-0.193). These data suggest electrophilic nitration should occur at the more electron-rich C-4 position, relative to C-5, on the phenyl moiety to give the 4,7-dinitro product.

Iodination of the 3,5-bis-*O*-(*p*-chlorobenzoyl) ester β-D-**5** using ICl in CH_2Cl_2 afforded the 7-iodo derivative **10** (87%), which on deprotection with NaOMe in MeOH gave 1-(2-deoxy-β-D-ribofuranosyl)-7-iodoisocarbostyril (**11**, 82%). Synthesis of the 7-trifluoromethyl derivative **13** was carried out using a reaction reported by Su *et al.* (1991). Thus, the protected 7-iodo compound **10** was treated with methyl chlorodifluoroacetate in the presence of CuI and KF in DMF at 120 °C to give the 3,5-bis-*O*-(*p*-chlorobenzoyl) ester **12** (71%), followed by deprotection with NaOMe in MeOH to afford 1-(2-deoxy-β-D-ribofuranosyl)-7-trifluoromethylisocarbostyril (**13**, 97%). The 7-cyano derivative **15** was also prepared from the protected 7-iodo derivative **10** by cyanation with zinc cyanide in the presence of $(PPh_3)_4Pd(0)$ (Tschaen *et al.*, 1994), and then deprotection using NaOMe in MeOH.

Synthesis of the (*E*)-7-(2-trimethylsilylvinyl) derivative **17** was performed using a methodology similar to that reported previously (Morin *et al.*, 1997) for the synthesis of (*E*)-5-(2-iodovinyl)-2'-fluoro-2'-deoxyuridine (IVFRU), as illustrated in Scheme 12. Accordingly, the coupling reaction of the unprotected 7-iodo compound **11** with (*E*)-1-trimethylsilyl-2-tributylstannylethene (**16**) (Cunico & Clayton, 1976) catalyzed by $(Ph_3P)_2PdCl_2$ afforded **17** (83%). The ^1H NMR spectrum for **17** showed a $J_{CH=CH}$ = 18.9

Hz coupling constant that is indicative of the (*E*)-7-(-CH=CH-TMS) stereochemistry. Reaction of the (*E*)-7-(2-trimethylsilylvinyl) compound **17** with ICl in MeCN at 0 °C yielded the corresponding (*E*)-7-(2-iodovinyl) product **18** (70%). Proton NMR spectral analysis of **18** indicated that only the (*E*)-isomer was produced ($J_{CH=CH}$ = 14.7 Hz).

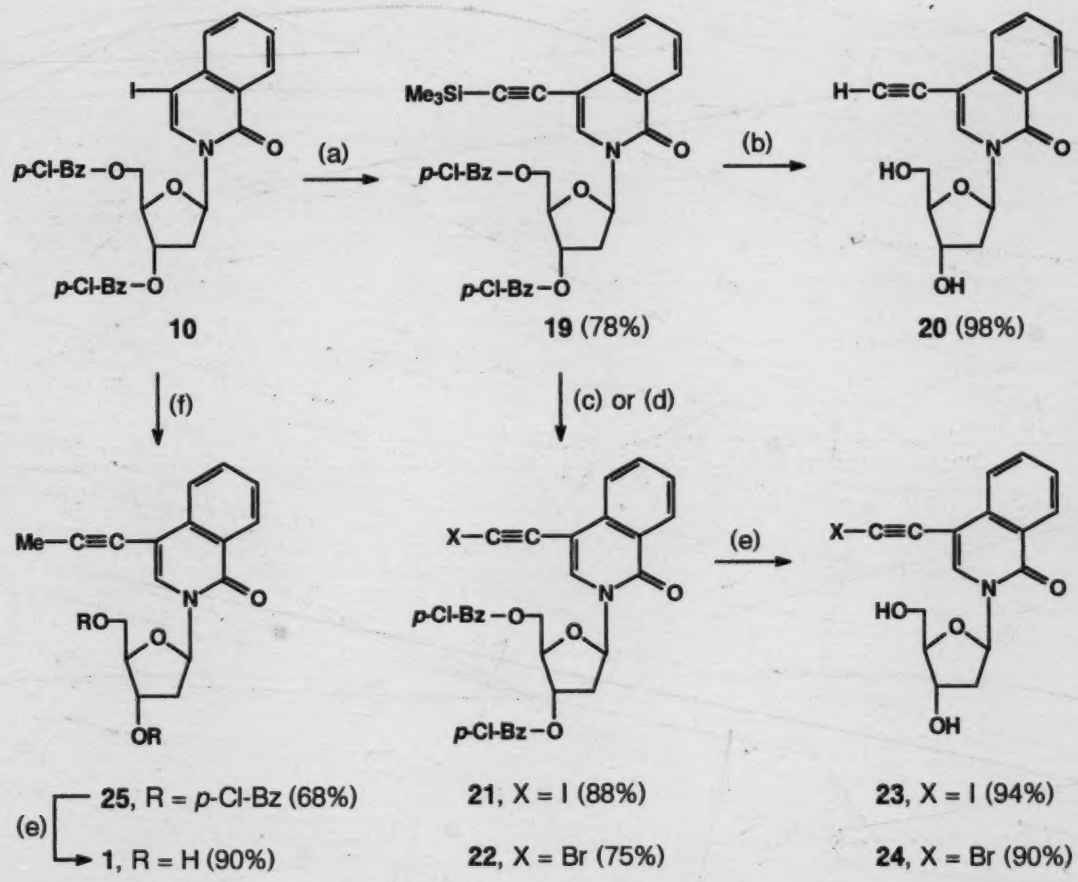


Scheme 12. Synthesis of 1-(2-deoxy-β-D-ribofuranosyl)-7-(*E*)-(2-iodovinyl) isocarbostyril (**18**).

Reagents and conditions: (a) $(Ph_3P)_2PdCl_2$, MeCN, 60 °C, 24 h; (b) ICl, MeCN, 0 °C, 15 min.

Reaction of 1-[3,5-bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl]-7-iodoisocarbostyril (**10**) with TMS-C≡C-H in the presence of $(Ph_3P)_2PdCl_2$ and CuI in Et_3N (Takahashi *et al.*, 1980) afforded the corresponding 7-(trimethylsilyl)ethynyl derivative **19** (78%), which upon treatment with NaOMe in MeOH at 20 °C resulted in simultaneous ester cleavage and removal of the TMS group to give the 7-ethynyl product **20** (98%) as illustrated in Scheme 13. The 7-iodoethynyl **23**, or 7-bromoethynyl **24**, compounds were prepared by reaction of the 7-(trimethylsilyl)ethynyl compound **19** with

either *N*-iodosuccinimide, or *N*-bromosuccinimide, in the presence of $AgNO_3$ catalyst in DMF (Nishikawa *et al.*, 1994), and then removal of the *p*-chlorobenzoyl protecting groups in the sugar moiety using NaOMe in MeOH.



Scheme 13. Synthesis of 1-(2-deoxy-β-D-ribofuranosyl)-7-substituted-isocarbostyrils **1**, **20**, **23** and **24**.

Reagents and conditions: (a) H-C≡C-$SiMe_3$, $(Ph_3P)_2PdCl_2$, CuI, Et_3N, 50-60 °C, 5 h; (b) NaOMe, MeOH, 25 °C, 20 min; (c) *N*-iodosuccinimide, $AgNO_3$, DMF, 0 °C for 2 h, and then 25 °C for 1 h; (d) *N*-bromosuccinimide, $AgNO_3$, DMF, 0 °C for 2 h, and then 25 °C for 4 h; (e) NaOMe, MeOH, 25 °C, 15 min; (f) $(Ph_3P)_2PdCl_2$, CuI, Et_3N, Me-C≡C-H, 25 °C, 5 h.

The coupling reaction of 1-[3,5-bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl]-7-iodoisocarbostyril (**10**) with Me-C≡C-H in the presence of $(Ph_3P)_2PdCl_2$, CuI and dry Et_3N, using a procedure similar to that reported by McMinn *et al.* (1999), and then ester hydrolysis using NaOMe in MeOH, afforded the target product 1-(2-deoxy-β-D-ribofuranosyl)-7-propynylisocarbostyril (**1**, 61% overall yield from **10**).

Biological results and discussion

Nucleoside mimics, in which the natural thymine base in thymidine is replaced by an unnatural isocarbostyril base, could induce new properties which the natural nucleosides lack thereby providing a strategy to design a new class of isocarbostyril nucleoside mimics. Isocarbostyril mimics possess a number of potentially desirable features that include i) irreversible inhibition of thymidylate synthase (TS), by the 5'-monophosphate, upon reaction of the nucleophilic –SH group of TS at the electron deficient C-8 position of the isocarbostyril ring to exhibit a cytotoxic anticancer effect, ii) utilization of the 5'-monosphosphate as a substrate for the synthesis of "false" DNA that would stop chain elongatation resulting in a cytotoxic antiviral/anticancer effect, and iii) increased lipophilicity which could enhance their ability to penetrate the blood-brain-barrier (BBB) to improve their efficacy for the treatment of brain viral infections and brain tumors. A group of 1-(2-deoxy-β-D-ribofuranosyl)-7-substituted-isocarbostyrils having a variety C-7 substituents [H, NO_2, I, CF_3, CN, (*E*)-CH=CH-I, -CH≡CH, -C≡C-I, -C≡C-Br, -C≡C-Me] were investigated to probe the effect of size, electronic, hybridization [sp (-C≡C-), sp^2 (-CH=CH-)] and lipophilic parameters upon biological activity. Substituent selection could also influence oral bioavailability, metabolic stability

and pharmacokinetic properties. All of these factors are potential determinants of antiviral/anticancer efficacy.

A group of 7-substituted [H, 4,7-$(NO_2)_2$, I, CN, (*E*)-(-CH=CH-I), -CH≡CH, -C≡C-I, -C≡C-Br, -C≡C-Me] isocarbostyril compounds, and the reference compounds 5-iodo-2'-deoxyuridine (IUDR), 5-fluoro-2'-deoxyuridine (FUDR) and thymidine, were evaluated using the MTT cytotoxicity assay (Alley *et al.*, 1988) (Table 3). These 7-substituted compounds exhibited weak cytotoxicity (CC_{50} = 10^{-3} to 10^{-5} M range), even when compared to thymidine (CC_{50} = 10^{-3} to 10^{-5} M range), against KBALB, KBALB-STK, 143B, 143B-LTK, EMT-6 and R-970-5 cancer cell lines. The 4,7-dinitro compound **9** was the most cytotoxic (CC_{50} = 10^{-5} M range) agent. A comparison of the cytotoxicities against the KBALB cell line showed the cytotoxicity potency order was 4,7-dinitro **9** ≥ 7-C≡C-I **23** > 7-C≡C-Br **24** ≈ 7-C≡CH **20** ≈ 7-I **11** ≈ 7-(*E*)-CH=CH-I **18** ≈ 7-CN **15** ≈ 7-C≡C-Me **1** > 7-H **7**. The I and Me groups are potential isosteres since their van der Waal's radii are 2.15 and 2.0 Å, respectively. This concept is supported by the observation that the cytotoxicity for 7-C≡C-I **23** is greater than 7-C≡C-Me **1** toward KBALB cells. The 4,7-dinitro **9**, 7-I **11** and 7-C≡CH **20** compounds exhibited similar cytotoxicity against non-transfected (KBALB, 143B), and the corresponding transfected (KBALB-STK, 143B-LTK) cancer cell lines possessing the herpes simplex virus type 1 (HSV-1) thymidine kinase gene (TK^+). This comparison, for these compounds, indicates that expression of the viral TK enzyme did not provide a gene therapeutic effect.

The weak, or absence of, anticancer/antiviral efficacy for this novel class of 1-(2-deoxy-β-D-ribofuranosyl)-7-substituted-isocarbostyrils could be due to a number of factors. For example, it is possible that the sugar moiety does not undergo

phosphorylation by thymidine kinase (TK) to the 5'-monophosphate (5'-MP) or the compounds are good substrates for the enzyme but are not good competitors for natural nucleosides. Support for this explanation is based on the observations that there are generally negligible differences in anticancer/antiviral activities between non-transfected (KBALB, 143B) and viral TK-transfected (KBALB-STK, 143B-LTK) cell lines.

Table 3. *In Vitro* Cell Cytotoxicity of 1-(2-Deoxy-β-D-ribofuranosyl)-7-substituted-isocarbostyrils Determined Using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium Bromide (MTT) Assay.

	Cellular toxicity (CC_{50}, M) toward various cell lines[a]					
Compd	KBALB[b]	KBALB-STK[c]	143B[d]	143B-LTK[c]	EMT-6[e]	R-970-5[f]
7	2.5×10^{-3}	—	—	—	—	—
9	2.7×10^{-5}	2.6×10^{-5}	3.2×10^{-5}	3.0×10^{-5}	2.0×10^{-5}	4.0×10^{-5}
11	3.3×10^{-4}	3.0×10^{-4}	2.6×10^{-4}	1.5×10^{-4}	2.8×10^{-4}	1.8×10^{-4}
15	5.5×10^{-4}	—	—	—	—	—
18	5.0×10^{-4}	—	—	—	—	—
20	3.1×10^{-4}	2.7×10^{-4}	2.6×10^{-4}	2.0×10^{-4}	2.3×10^{-4}	2.3×10^{-4}
23	7.5×10^{-5}	—	—	—	—	—
24	2.0×10^{-4}	—	—	—	—	—
1	5.5×10^{-4}	—	—	—	—	—
IUDR[g]	9.7×10^{-5}	1.0×10^{-5}	7.0×10^{-3}	7.4×10^{-3}	3.8×10^{-4}	6.2×10^{-4}
FUDR[h]	6.0×10^{-11}	8.8×10^{-11}	9.0×10^{-5}	1.0×10^{-4}	9.0×10^{-12}	5.5×10^{-5}
Thymidine	9.5×10^{-5}	1.0×10^{-4}	—	—	1.3×10^{-4}	2.5×10^{-3}

[a]The molar concentration of the test compound that killed 50% of the cells (or 50% cell survival) upon incubation for 3-5 days at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 (Mean value, n = 6). [b]Transformed fibroblast sarcoma cell line. [c]These cells were transfected by, and expressed, the herpes simplex virus type 1 thymidine kinase (HSV-1 TK) gene. [d]Human osteosarcoma cell line. [e]Mouse mammary carcinoma cell line. [f]Human osteosarcoma cell line. [g]IUdR = 5-iodo-2'-deoxyuridine. [h]FUdR = 5-fluoro-2'-deoxyuridine.

In this respect, one would anticipate that preferential phosphorylation to the 5'-MP by transfected cells, and subsequent transformation to the active 5'-triphosphate (5'-TP), would have resulted in a greater cytotoxic effect since the 5'-TP of **1** has been shown to deter chain elongation (McMinn *et al.*, 1999). Alternatively, the group of nucleoside mimics evaluated may be devoid of anticancer/antiviral activity due to the fact that they are not inhibitors of thymidylate synthase (TS). Credence for this possibility is based on the belief that the anticancer activity exhibited by 5-fluoro-2'-deoxyuridine is primarily due to inhibition of DNA biosynthesis by blocking TS, the enzyme which catalyzes the methylation of 2'-deoxyuridine-5'-monophosphate to thymidine-5'-monophosphate (Langenbach *et al.*, 1972; Santi *et al.*, 1974). Furthermore, inhibition of TS is the mechanism by which certain nucleosides such as (*E*)-5-(2-iodovinyl)-2'-deoxyuridine and 5-(1-azidovinyl)-2'-deoxyuridine exhibit their cytostatic effect (Balzarini *et al.*, 1995).

2. Synthesis of 3'- and 5'-nitrooxy pyrimidine nucleosides as NO-donor/nucleoside hybrid drugs for evaluation as anticancer and antiviral agents

Organic nitrates ($RONO_2$), which are nitric acid esters of mono- or polyhydric alcohols, represent the oldest class of NO-donor compounds that include the classical drug glycerol trinitrate (**26**) that is used to treat angina pectoris (Wang *et al*, 2002). Although some 5'-*O*-nitro-2'-deoxyuridine (**27**) (Lichtenthaler & Muller, 1973; Schwandt *et al.*, 1968), 3',5'-di-*O*-nitro-2'-deoxyuridine (**28**) (Duschinsky & Eppenberger, 1967; Duschinsky, 1978; Giziewicz *et al.*, 1999), and 3'-*O*-nitro-2'-deoxycytidine (**29**) (Huang & Torrence, 1978) compounds have been synthesized (Figure

29), no biological data were reported. On the other hand, the 2'-*O*-nitro derivatives of arabinocytidine (Nitrara-C, **30**), which resists enzymatic deamination, exhibited antileukemic activity *in vitro* and *in vivo* (Chwang *et al.*, 1983). The related 2'-*O*-nitro derivative of arabinoadenine (Nitrara-A, **31**), that is deaminated about 25-fold slower than Ara-A, inhibited the proliferation of human T-lymphoblasts (CCRF-CEM cell line) *in vitro* (Chwang *et al.*, 1983).

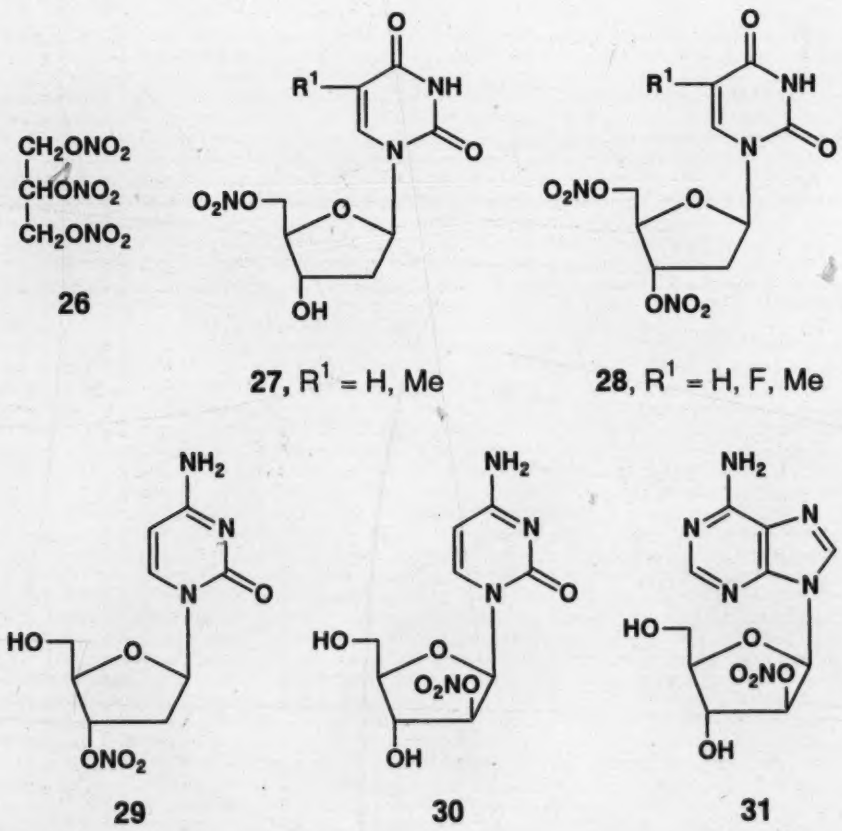


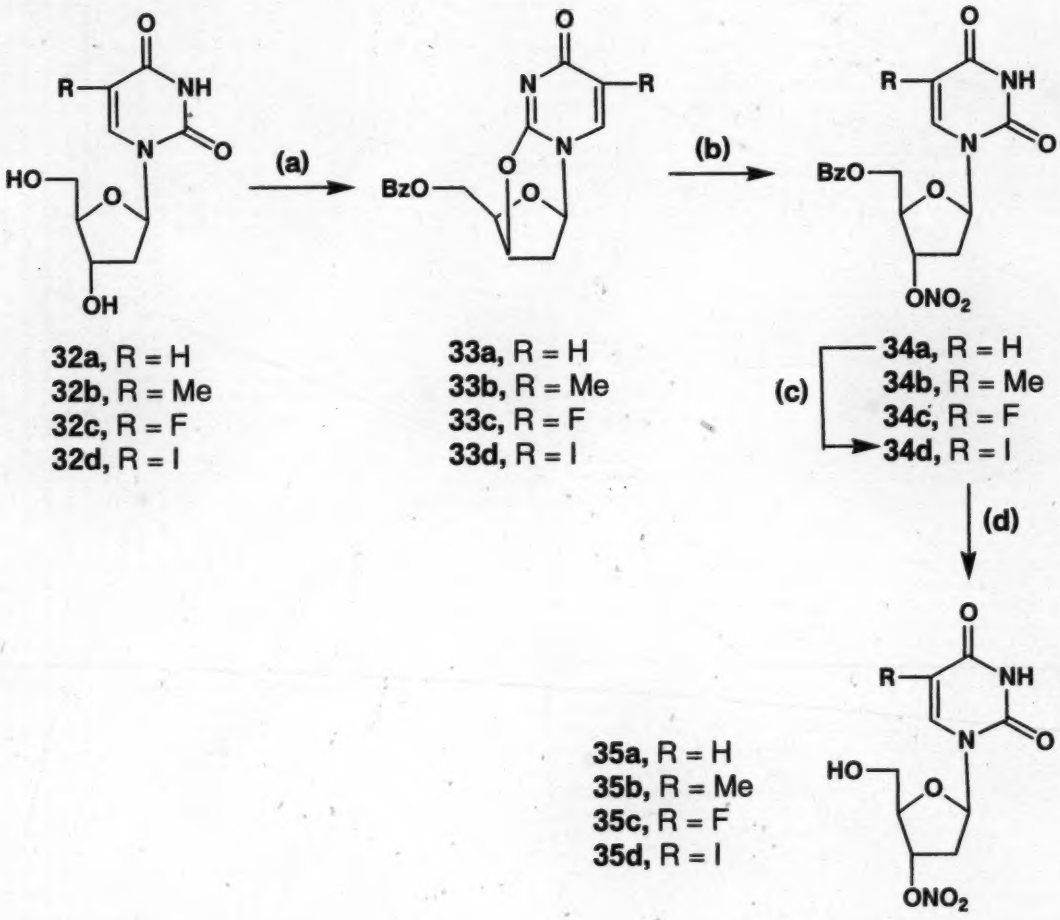
Figure 29. Structures of glycerol trinitrate (**26**), 5'-*O*-nitro-2'-deoxyuridines (**27**), 3',5'-di-*O*-nitro-2'-deoxyuridines (**28**), 3'-*O*-nitro-2'-deoxycytidine (**29**), nitrara-C (**30**) and nitrara-A (**31**) nitrate esters.

The design of tumor selective NO-donor/nucleoside hybrid drugs, that release both cytotoxic NO and a nucleoside drug in a controlled fashion, constitutes an attractive method to develop a novel class of double-barreled anticancer drugs. The nucleoside component of these hybrid drugs, as the 5'-monophosphate, could act as an irreversible inhibitor of thymidylate synthase (TS), whereas the 5'-triphosphate could serve as an unnatural substrate for, or inhibitor of, DNA polymerase to elicit a cytotoxic anticancer/antiviral effect. The syntheses, *in vitro* NO release data, and anticancer/antiviral activities, for a group of 3'-*O*-nitro-2'-deoxyuridines (**35a-d**), 3'-*O*-nitro-2'-deoxycytidines (**38a-c**), and 5'-*O*-nitro-2'-deoxyuridines (**40a-c**) are now reported.

Chemistry

A group of 2,3'-anhydro-5'-*O*-benzoyl-2'-deoxyuridines **33a-d** possessing a variety of C-5 substituents (R = H, Me, F, I) were prepared from the 2'-deoxyuridine precursors **32a-d** by a one-pot tandem Mitsunobu reaction (Czernecki & Valery, 1990; Becouarn *et al.*, 1995), using triphenylphosphine, diisopropyl azodicarboxylate, and benzoic acid in 81%, 74%, 90%, and 82% yield, respectively. Ring opening of the 2,3'-anhydro derivatives **33a-d** proceeded readily upon reaction with ammonium nitrate (NH_4NO_3) in dimethyl formamide to form the respective 3'-*O*-nitrate esters **34a-d** in 31%, 34%, 50% and 2.8% yield, respectively (Scheme 14). Since conversion of the 5-iodo compound **33d** to **34d** was low (2.8% yield) in this reaction, compound **34d** was prepared by iodination of **34a** using iodine monochloride (ICl) and sodium azide (NaN_3) in acetonitrile (Kumar *et al.*, 1994) in 82.5% yield. Deprotection of compounds **34a-d**

using NaOMe in MeOH afforded the target 5-substituted-3'-*O*-nitro-2'-deoxyuridines **35a-d** in 91-95% yield.



Scheme 14. Synthesis of 5-substituted-3'-*O*-nitro-2'-deoxyuridines **35a-d** using the Mitsunobu reaction.

Reagents and conditions: (a) C_6H_5COOH, Ph_3P, DIAD, DMF, 25 °C; (b) NH_4NO_3, DMF, 110 °C; (c) ICl, NaN_3, MeCN, 0 °C → 25 °C; (d) NaOMe, MeOH, 25 °C.

The configuration at the C-3' carbon, and the rotamer orientation of the uracil **(35a)** and thymine **(35b)** ring, was analyzed by nuclear Overhauser enhancement (NOE) ^{1}H NMR difference spectroscopy (Figure 30).

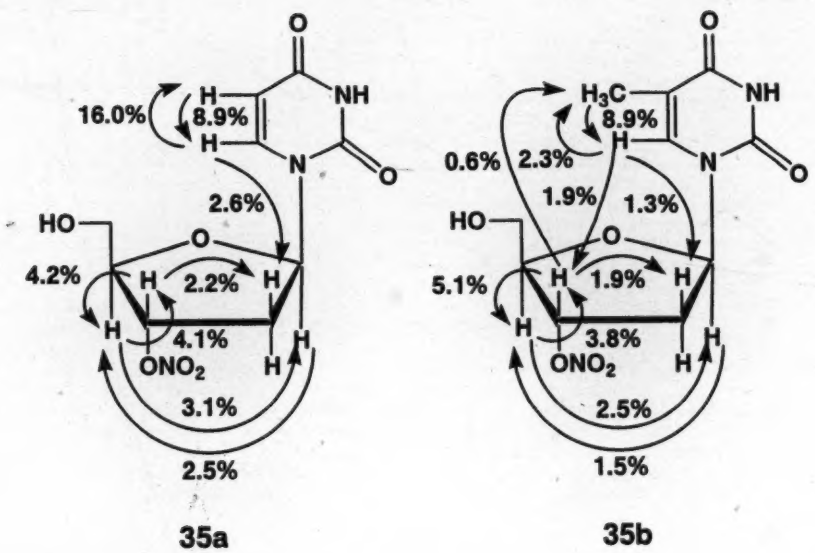
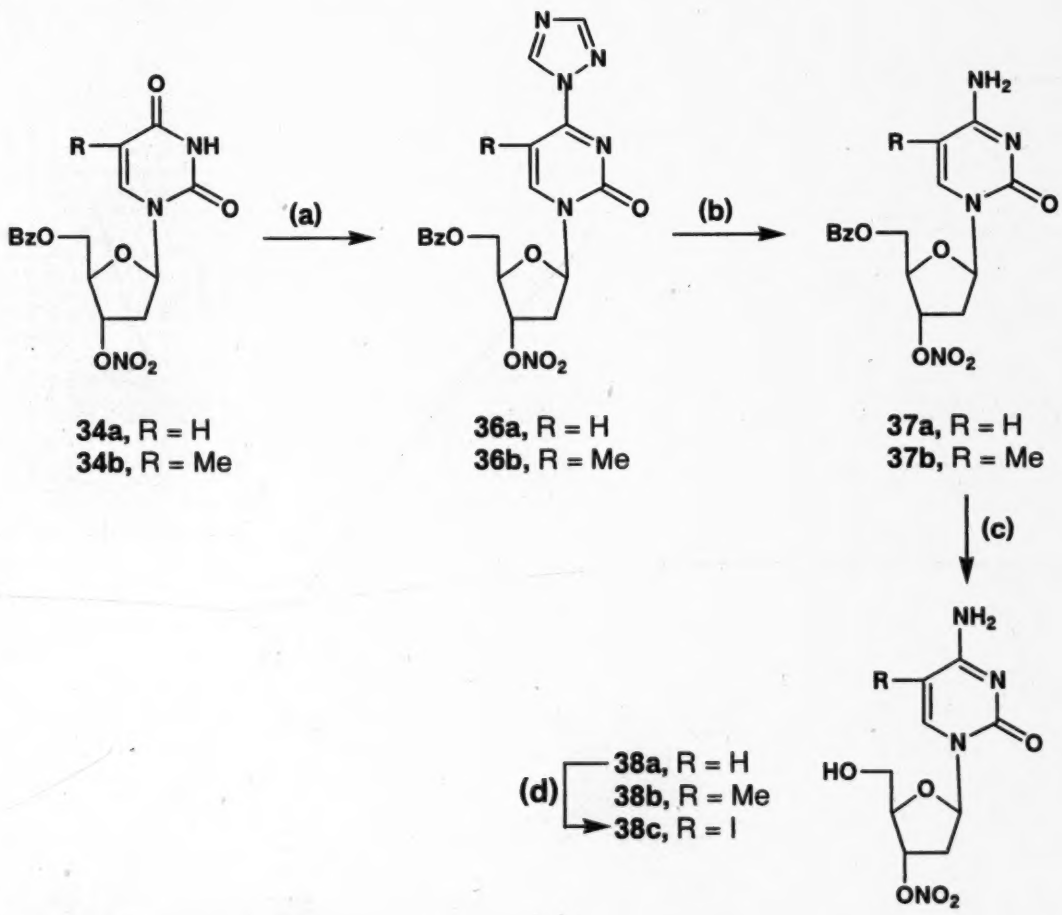


Figure 30. Some NOE measurements to determine the rotameric orientation of the uracil base moiety, and the conformation of the nitrooxy substituent, for the 3'-*O*-nitro-2'-deoxyuridine compounds **35a-b** in DMSO-d_6 at 22 °C.

Selective irradiation of the H-3' signal resulted in an enhancement of the arabino (up) H-2' signal of **35a** (2.2%), and of **35b** (1.9%), indicating that the C-3' nitrooxy substituent is in the ribo (down) position. The observation that selective irradiation of the H-4' signal resulted in enhancement of the H-1' signal of **35a** (3.1%), and of **35b** (2.5%), indicates that these two hydrogen atoms are on the same face of the sugar moiety which is indicative of the β-configuration (Walker *et al.*, 1997). Selective irradiation of the H-6 signal of **35a** produced an enhancement of the arabino (up) H-2' signal (2.6%), whereas a similar irradiation for **35b** showed enhancement for the arabino H-2' (1.3%), and the H-3' (1.9%), signals. These NOE data indicate the C-6 hydrogen of the uracil moiety of **35a**, or the thymine moiety of **35b**, is oriented in the direction of the sugar ring.

A related group of 3'-*O*-nitro-2'-deoxycytidines **38a-c** having a C-5 hydrogen, methyl, or iodo substituent were prepared using the three-step reaction sequence illustrated in Scheme 15.



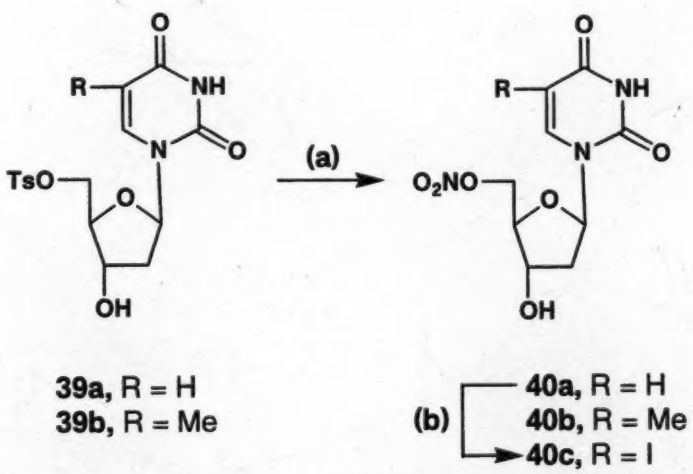
Scheme 15. Synthesis of 5-substituted-3'-*O*-nitro-2'-deoxycytidines **38a-c**. Reagents and conditions: (a) 4-chlorophenyl dichlorophosphate, 1,2,4-triazole, pyridine, 0 °C → 25 °C; (b) NH_4OH, dioxane, 25 °C; (c) NaOMe, MeOH, 25 °C; (d) iodine, iodic acid, acetic acid, H_2O, CCl_4, 25 °C.

Thus, reaction of the 5'-*O*-benzoyl-3'-*O*-nitro-2'-deoxyuridines **34a-b** with 1,2,4-triazole and 4-chlorophenyl dichlorophosphate in pyridine (Sung, 1981; Huang *et al.*,

1991; Kozlov *et al.*, 1998) afforded the corresponding 4-(1,2,4-triazolo) derivatives **36a** and **36b** in 79 and 71% yield, respectively. Subsequent treatment of **36a**, or **36b**, with aqueous ammonia in dioxane (Sung, 1981; Huang *et al.*, 1991; Kozlov & Orgel, 1998) yielded the 2'-deoxycytidine compound **37a** (49%), or **37b** (36%), which on deprotection with NaOMe in MeOH to remove the 5'-O-benzoyl moiety, afforded the target 3'-*O*-nitro-2'-deoxycytidine **38a** (95%), or 5-methyl-3'-*O*-nitro-2'-deoxycytidine **38b** (94%), respectively. Iodination of **38a** with iodic acid and iodine (Irani & Santa Lucia, 1999; Bobek *et al.*, 1987) yielded 5-iodo-3'-*O*-nitro-2'-deoxycytidine **38c** (56%). The procedure described above for the synthesis of **38a** is superior to the reported method involving nitration of 2'-deoxycytidine 5'-*O*-monophosphate using nitronium tetrafluoroborate, which gave a mixture of the 5'-*O*-monophosphates of 5-nitro-3'-*O*-nitro-2'-deoxycytidine, 5-nitro-2'-deoxycytidine, and 3'-*O*-nitro-2'-deoxycytidine in a ratio of 12:1:3 that were subsequently dephosphorylated by the action of alkaline phosphatase to the free nucleosides (Huang & Torrence, 1978).

The 5'-*O*-nitro-2'-deoxyuridines **40a-c** possessing a C-5 H, Me or I substituent were also prepared (Scheme 16). Accordingly, nucleophilic displacement of the 5'-*O*-tosylate moiety in **39a**, or **39b**, using $LiNO_3$ in DMF at 110 °C afforded the respective 5'-*O*-nitro product **40a** (73%), or **40b** (71%). This synthesis of **40a** was superior to nitration of 2'-deoxyuridine with 90% nitric acid at –70 °C which gave a mixture of **40a** (20%) and 3',5'-di-*O*-nitro-2'-deoxyuridine (13%) (Lichtenthaler & Muller, 1973). Synthesis of **40b** by displacement of the 5'-O-tosylate moiety in **39b**, was equally effective to a reported method involving displacement of either a 5'-*O*-P(=S)OEt_3, or 5'-I, substituent

using $AgNO_3$ (Schwandt *et al.*, 1968). Electrophilic iodination of **40a** using ICl and NaN_3 in MeCN gave the 5-iodo product **40c** (65%).



Scheme 16. Synthesis of 5-substituted-5'-*O*-nitro-2'-deoxyuridines **40a-c**. Reagents and conditions: (a) $LiNO_3$, DMF, 100-110 °C, overnight; (b) ICl, NaN_3, MeCN, 0 °C → 25 °C, 48 h.

In vitro NO release evaluation

Organic nitrate esters are generally stable in neutral or mild acidic aqueous solution, but nucleophilic hydrolysis to an alcohol and nitrate occur under strongly alkaline conditions. NO release from organic nitrates may occur as a result of either non-enzymatic or enzymatic biotransformation that proceeds via a three-electron reduction. Accordingly, cellular thiols may play a role in the non-enzymatic production of NO from glycerol trinitrate (**26**, GTN) whereas, a NADPH-dependent cytochrome P450 pathway and specific isozymes of the glutathione-*S*-transferase group are thought to be operative in the biotransformation/bioactivation of organic nitrates (Wang *et al.*, 2002). Although

the sole product from reaction of NO with water is nitrite, NO reacts rapidly with superoxide anion to initially produce a cytotoxic peroxynitrite anion ($ONOO^-$) species that can damage DNA (Kerwin *et al.*, 1995).

The percentages of NO released, quantitated as nitrite (NO_2^-) using the Griess reaction (Sako *et al.*, 1998), upon incubation of the 3'-*O*-nitro derivatives of 2'-deoxyuridine (**35a-d**) and 2'-deoxycytidine (**38a-c**), and the 5'-*O*-nitro derivatives of 2'-deoxyuridine (**40a-c**) in the presence of 18 mM L-cysteine or serum are listed in Table 4.

Table 4. *In Vitro* Percent Nitric Oxide Release for 3'-*O*-nitro-2'-deoxyuridines (**35a-d**), 3'-*O*-nitro-2'-deoxycytidines (**38a-c**) and 5'-*O*-nitro-2'-deoxyuridines (**40a-c**).

	% NO release[a] (18 mM L-cysteine)		% NO release[b] (serum)	
compd	1 hour	16 hours	1 hour	16 hours
35a	4.9 ± 0.0	51.0 ± 0.1	7.7 ± 0.7	9.0 ± 1.5
35b	4.9 ± 0.1	53.6 ± 0.7	7.4 ± 0.9	7.4 ± 0.3
35c	0.0 ± 0.0	3.8 ± 0.1	4.0 ± 0.9	6.6 ± 0.7
35d	3.9 ± 0.1	37.6 ± 0.1	12.3 ± 1.1	13.2 ± 0.3
38a	1.5 ± 0.0	17.1 ± 0.2	18.8 ± 0.9	20.2 ± 0.9
38b	3.8 ± 0.0	50.3 ± 0.7	47.1 ± 3.1	59.5 ± 1.5
38c	4.0 ± 0.1	47.2 ± 0.0	12.5 ± 0.5	17.6 ± 1.7
40a	5.4 ± 0.1	59.9 ± 1.7	2.9 ± 0.8	4.4 ± 1.0
40b	5.0 ± 0.0	59.8 ± 0.2	3.6 ± 0.4	4.8 ± 1.1
40c	3.7 ± 0.1	36.4 ± 0.4	3.9 ± 0.5	4.7 ± 1.0
ISDN[c]	3.5 ± 0.2	24.0 ± 0.2	1.9 ± 0.1	2.6 ± 0.6

[a]The percent nitric oxide released from the test compound was determined as the percent of nitrite (NO_2^-) produced in the presence of L-cysteine (18 mM) as quantitated using the Griess reagent (± SD, n = 3).

[b]The percent nitric oxide released from the test compound was determined as the percent of nitrite (NO_2^-) produced in the presence of serum as quantitated using the Griess reagent (± SD, n = 3).

[c]ISDN = isosorbide dinitrate (ISDN possesses two ONO_2 groups which may release NO, whereas compounds **35**, **38** and **40** possess only one ONO_2 group that may release NO).

All three groups of compounds (**35**, **38**, **40**) that possess a single ONO_2 moiety, with the exception of 5-fluoro-3'-*O*-nitro-2'-deoxyuridine (**35c**), exhibited comparable or in most instances superior nitric oxide release (1.5-5.4% range at 1 hour, and 17.1-59.9% range at 16 hours) upon incubation in the presence of L-cysteine compared to the reference drug isosorbide dinitrate (3.5 and 24% release at 1 hour and 16 hours) that contains two ONO_2 moieties. These results are consistent with the observation that thiols enhance in vitro release of NO in aqueous buffer solution for NO-donor compounds although intact cells and tissues show that conversion of organic nitrate esters ($RONO_2$) to NO is not free-thiol dependent (Gasco *et al.*, 1996). In contrast, release of NO from **35**, **38** and **40** was negligible in the absence of L-cysteine (< 0.05% at 1 hour and < 1.0% at 16 hours). A similar study to determine the release of NO upon incubation in the presence of serum showed that 3'-*O*-nitro-2'-deoxycytidine compounds (**38**) generally provided a greater percent NO release than the related compounds **35** and **40**. Compounds **35a-d**, **38a-c** and **40a-c** all exhibited a greater release of NO in the presence of serum (2.9-47.1% range at 1 hour, and 4.4-59.5% range at 16 hours) than the reference drug isosorbide dinitrate (1.9% at 1 hour and 2.6% at 16 hours).

Anticancer and antiviral activity evaluation

Compounds **35a-d**, **38a-c**, **40a-c**, and the reference compounds 5-iodo-2'-deoxyuridine (IUDR), 5-fluoro-2'-deoxyuridine (FUDR) and thymidine, were evaluated for their tumor cell cytotoxicity using the MTT cytotoxicity assay (Alley et al., 1988) (see data in Table 5). These 3'- and 5'-*O*-nitro pyrimidine nucleosides exhibited comparable cytotoxicity (CC_{50} = 10^{-3} to 10^{-6} M range) to 5-iodo-2'-deoxyuridine, but weak

cytotoxicity in comparison to 5-fluoro-2'-deoxyuridine, against KBALB, KBALB-STK, 143B, 143B-LTK and EMT-6 cell lines. 5-Fluoro-3'-*O*-nitro-2'-deoxyuridine (**35c**) was the most cytotoxic compound against this group of cancer cell lines (CC_{50} = 10^{-6} M range).

Table 5. *In Vitro* Cell Cytotoxicity for 3'-*O*-nitro-2'-deoxyuridines (**35a-d**), 3'-*O*-nitro-2'-deoxycytidines (**38a-c**) and 5'-*O*-nitro-2'-deoxyuridines (**40a-c**).

	Cellular toxicity (CC_{50}, M) toward various cell lines[a]				
compd	KBALB[b]	KBALB-STK[c]	143-B[d]	143B-LTK[c]	EMT-6[e]
35a	–	–	–	8.5×10^{-4}	–
35b	1.0×10^{-3}	7.0×10^{-3}	–	–	–
35c	8.0×10^{-6}	3.5×10^{-6}	2.4×10^{-6}	7.4×10^{-5}	6.2×10^{-6}
35d	2.4×10^{-4}	2.6×10^{-4}	5.8×10^{-4}	4.1×10^{-4}	2.3×10^{-5}
38a	–	–	–	2.0×10^{-4}	–
38b	–	–	–	1.0×10^{-3}	–
38c	1.0×10^{-3}	2.5×10^{-4}	2.0×10^{-4}	2.0×10^{-4}	3.4×10^{-4}
40a	1.0×10^{-3}	–	–	–	–
40b	–	3.0×10^{-4}	–	–	–
40c	3.4×10^{-4}	2.5×10^{-4}	3.4×10^{-4}	2.1×10^{-5}	–
IUDR[f]	9.7×10^{-5}	1.0×10^{-5}	7.0×10^{-3}	7.4×10^{-3}	3.8×10^{-4}
FUDR[g]	6.0×10^{-11}	8.8×10^{-11}	9.0×10^{-5}	1.0×10^{-4}	9.0×10^{-12}
Thymidine	9.5×10^{-5}	1.0×10^{-4}	–	–	1.3×10^{-4}

[a]The molar concentration of the test compound that killed 50% of the cells (or 50% cell survival) upon incubation for 3-5 days at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 (mean value, n = 6) was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. [b]Transformed fibroblast sarcoma cell line. [c]These cells were transfected by, and expressed, the herpes simplex virus type 1 thymidine kinase (HSV-1 TK) gene. [d]Human osteosarcoma cell line. [e]Mouse mammary carcinoma cell line. [f]IUDR = 5-iodo-2'-deoxyuridine. [g]FUDR = 5-fluoro-2'-deoxyuridine.

Elaboration of the 3'-OH group of IUDR to a 3'-*O*-NO_2 group (**35d**) decreased cytotoxicity by 2.5- to 26-fold in all cell lines except for EMT-6 cells where cytotoxicity

was enhanced by about 6-fold. The position of the *O*-NO_2 substituent was not a determinant of anticancer activity, since 5-iodo-3'-*O*-nitro-2'-deoxyuridine (**35d**) and 5-iodo-5'-*O*-nitro-2'-deoxyuridine (**40c**) were approximately equipotent. The observation that compounds **35c-d** and **38c** exhibited similar cytotoxicity against non-transfected (KBALB, 143B), and the corresponding transfected (KBALB-STK, 143B-LTK) cancer cell lines possessing the herpes simplex virus type 1 (HSV-1) thymidine kinase gene (TK^+) indicates that expression of the viral TK enzyme did not provide a gene therapeutic effect. This observation was also made for **35a** and **35b** that did not show increased cytostatic activity in murine mammary (FM3A) and human osteosarcoma cells transfected with the HSV-1 TK gene when compared with their parental counter-parts (data not shown).

Compounds **35a-d** were evaluated for their antiviral activity in a wide variety of assay systems (De Clercq, 1994). Antiviral activities against herpes simplex virus type 1 (KOS), herpes simplex virus type 2 (G), thymidine kinase-deficient (TK^-) herpes simplex virus type 1 (KOS, ACV^r), vaccinia virus, and vesicular stomatitis virus in E_6SM cells were determined. These compounds were generally inactive, or exhibited negligible activity in these antiviral assay systems (at concentrations up to 400 μg/mL). 5-Iodo-3'-*O*-nitro-2'-deoxyuridine (**35d**) showed some inhibitory activity against HSV-1 (KOS), HSV-2 (G), and vaccinia virus (IC_{50} = 48 μg/mL). In addition, compounds **35a-d** did not reduce cytopathicity induced by parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsakie B4 virus or Punta Toro virus in Vero cell cultures (IC_{50} > 80 μg/mL), or respiratory syncytial virus in HeLa cell cultures (IC_{50} > 80 μg/mL).

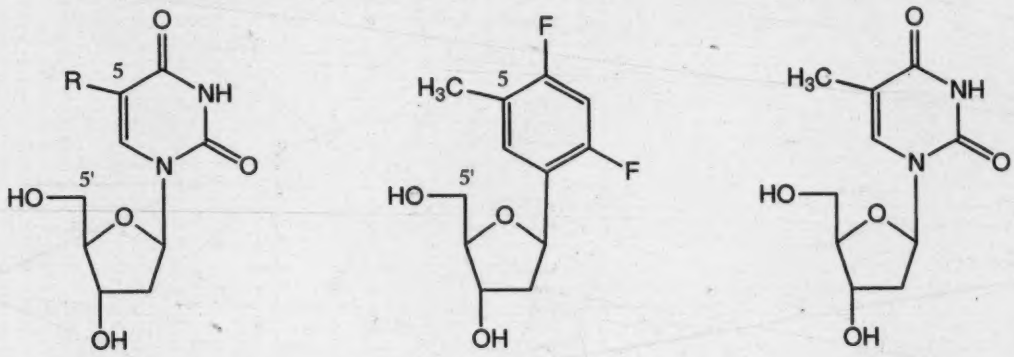
The modest anticancer, and near absence of antiviral, efficacy for this novel class of NO-donor nucleosides could be due to a number of factors. For example, it is possible that the sugar moiety does not undergo phosphorylation by thymidine kinase (TK) to the 5'-monophosphate (5'-MP). Support for this explanation is based on the observations that there are generally negligible differences in anticancer/antiviral activities between non-transfected (KBALB, 143B) and viral TK-transfected (KBALB-STK, 143B-LTK) cell lines. Alternatively, this group of nucleoside nitrate esters may be devoid of anticancer/antiviral activity due to the fact that they are not inhibitors of thymidylate synthase (TS). Credence for this possibility is based on the belief that the anticancer activity exhibited by 5-fluoro-2'-deoxyuridine is primarily due to inhibition of DNA biosynthesis by blocking TS, the enzyme which catalyzes the methylation of 2'-deoxyuridine-5'-monophosphate to 2'-deoxythymidine-5'-monophosphate (Langenbach *et al.*, 1972; Santi *et al.*, 1974). Also, according to our unpublished work compound **35d** (3'ONO_2-IUDR) was higly deiodinated to **35a** (3'ONO_2-UDR) in rat model. Therefore, it is possible that 3'ONO_2-UDR by releasing NO is converted to UDR, which is nontoxic.

3. Synthesis of 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-(2-halo-1-hydroxyethyl)benzenes and related derivatives: "thymine replacement" analogs of deoxythymidine for evaluation as antiviral and anticancer agents

The discovery that 2'-deoxyuridines possessing novel 2-carbon substituents at the C-5 position, are potent and selective antiviral agents, represented a significant advance in antiviral drug design. Accordingly, the development of (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU, Brivudin), (*E*)-5-(2-bromovinyl)arabinouridine (BVaraU,

Brovavir) and 5-(prop-1-ynyl)arabinouridine (PAU, Zonavir) significantly enhanced antiviral specificity since earlier antiherpetics, such as 5-iodo- and 5-trifluoromethyl-2'-deoxyuridine, exhibited little if any selectivity in their antiviral action. BVDU, BVaraU and PAU are highly specific inhibitors of herpes simplex virus (HSV-1 and varicella-zoster virus (VZV) replication due to their selective phosphorylation by the virus-encoded thymidine kinase (TK) in virus-infected, but not in normal, host cells (De Clercq, 1983; De Clercq, 1986; Sorivudine 1995; Kinchington & Goldthorpe, 1995]. Among the many 5-substituted pyrimidine nucleosides that have been investigated, (*E*)-5-[2-halo (iodo, IVDU; bromo, BVDU; chloro, CVDU]-vinyl (De Clercq, 1983; De Clercq, 1986) and 5-(2-chloroethyl)-2'-deoxyuridine (CEDU) (Griengl *et al.*, 1985) are among the most potent and selective in their action against HSV-1. CEDU is effective against systemic HSV-1 infection and HSV-1 encephalitis in mice at a 5-15-fold lower dose than BVDU (De Clercq & Rosenwirth, 1985). On the other hand, the less potent 5-ethyl-2'-deoxyuridine (EDU) is approximately equi-active against HSV-1 and HSV-2 (De Clercq, 1983; De Clercq, 1986). In earlier studies, it's shown that 2'-deoxyuridines (**41**) possessing novel C-5 $-CH(OH)CH_2X$, $-CH(OMe)CH_2X$, $-CH(OH)CHX_2$ and $-CH(OMe)CHX_2$ (X = I, Br, Cl) substituents (Figure 31) exhibited *in vitro* antiviral (HSV-1), and cytotoxic (L1210), activity (Kumar *et al.*, 1989; Kumar *et al.*, 1990; Kumar *et al.*, 1994). Modification of pyrimidine nucleoside bases can induce new, or improved, oral bioavailability, metabolic stability and pharmacokinetic properties, which the natural bases lack (Perigaud *et al.*, 1992). Consequently, their incorporation into nucleic acids by enzymatic processes can change the structure and/or function of these biopolymers. Some *C*-nucleosides, with a structural resemblance to natural *N*-nucleosides, exhibit anticancer

and antiviral activities (Hacksell & Daves, 1985; Montgomery, 1989; Mansuri & Martin, 1988). In this respect, nonpolar hydrophobic isosteres of pyrimidine nucleosides which have close structural, steric and isoelectronic relationships to the natural base have been reported (Schweitzer & Kool, 1994). For example, the 2,4-difluoro-5-methylphenyl isostere (**42**) (Figure 31) was designed as an unnatural mimic of thymidine (**43**).



41 **42** **43**

Figure 31. Structures of C-5 substituted $-CH(OH)CH_2X$, $-CH(OMe)CH_2X$, $-CH(OH)CHX_2$ and $-CH(OMe)CHX_2$ (X = I, Br, Cl) 2'-deoxyuridines (**41**), 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-methylbenzene (**42**) and thymidine (**43**).

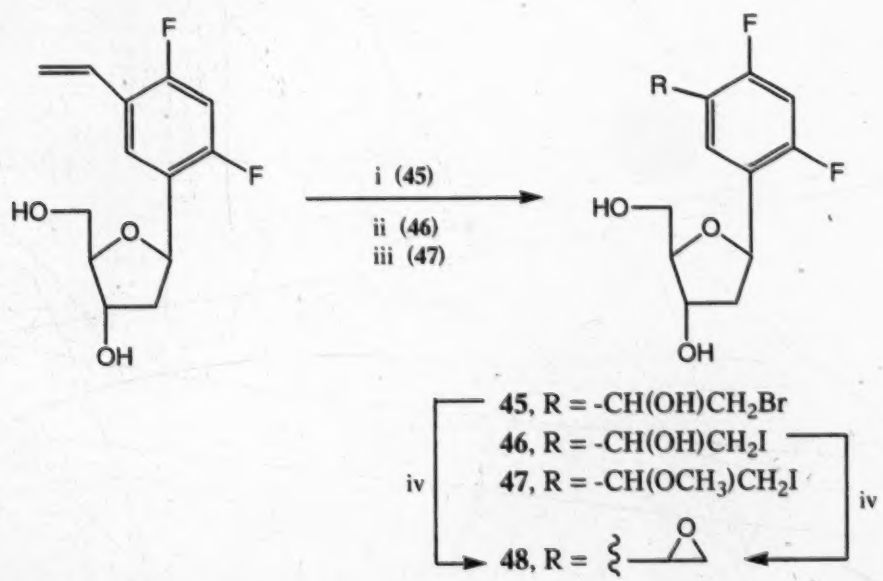
The 5'-triphosphate of **42** (**42**-TP) is incorporated into replicating DNA strands by the Klenow fragment (KF, exo$^-$ mutant) of *E. coli* DNA polymerase 1 (Moran *et al.*, 1997). Steady-state measurements indicated that **42**-TP is inserted opposite adenine (A) with an efficacy (V_{max}/K_m) only 40-fold lower than the triphosphate of thymidine (dTTP). Furthermore, it was inserted opposite A, relative to C, G, or T, with a selectivity almost as high as that for dTTP. Therefore, the 2,4-difluoro-5-methylphenyl moiety of **42** is a shape mimic used by KF polymerase like the natural thymine base that it replaces (Moran *et al.*, 1997; Morales & Kool, 1998; Guchian *et al.*, 1998). Accordingly, it was anticipated

that nucleoside mimic derivatives of **42**, possessing appropriate C-5 two-carbon substituents, may be cytotoxic to rapidly multiplying cancer cells (inhibit tumor growth) and/or act as antiviral agents due to their selective phosphorylation by virus-infected cells (De Clercq, 1984), and as chemotherapeutic agents for treating (Oldfield *et al.*, 1993), herpes simplex virus type-1 thymidine kinase positive (HSV-1 TK^+) gene-transfected tumors (gene therapy of cancer) (Wiebe *et al.*, 1997). Therefore, the synthesis, antiviral and anticancer activities for a group of 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-substituted-benzenes (**45-48, 51-52**) designed as 5-substituted-2'-deoxyuridine (thymidine) mimics is reported herein.

Chemistry

The 5-(2-bromo-1-hydroxyethyl)- derivative of 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluorobenzene (**45**) was synthesized by reaction of the 5-vinyl derivative (**44**) with *N*-bromosuccinimide (NBS) in aqueous dioxane in 76% yield, as illustrated in Scheme 17. The related 5-(1-hydroxy-2-iodoethyl) analog (**46**) could not be prepared in this way due to the instability of *N*-iodosuccinimide in aqueous dioxane. Accordingly, the 5-(1-hydroxy-2-iodoethyl) compound (**46**) was synthesized in 71% yield by reaction of the 5-vinyl precursor (**44**) with iodine in the presence of the oxidizing agent iodic acid (Cornforth & Green, 1970). The ^{13}C NMR (*J* modulation) spectra for **45** and **46** provided conclusive evidence for the regiospecific addition of HOBr and HOI to the C-5 vinylic substituent (**44**). The ^{13}C NMR spectrum for the 5-(2-bromo-1-hydroxyethyl) compound (**45**), showed the bromine atom is attached to a methylene carbon that exhibited dual resonances at δ 38.00 and 38.07, whereas the hydroxyl substituent is attached to a

methine carbon that exhibited dual resonances at δ 65.89 and 66.08. By analogy, for the 5-(1-hydroxy-2-iodoethyl) compound (**46**), the iodine atom is attached to a methylene carbon that showed dual resonances at δ 12.58 and 13.06, while the hydroxyl substituent is attached to a methine carbon that showed dual resonances at δ 65.97 and 66.39. Both compounds **45** and **46** exist as a mixture of two diastereomers, in a ratio of about 1:1, which differ in configuration (R and S) at the C-1 position of the 5-(2-halo-1-hydroxyethyl) moiety. This regiospecific addition is consistent with literature data in which unsymmetrical olefins capable of bromonium (halonium) ion formation were found to favor an unsymmetrical bridged intermediate even in solvents having a high dipole moment (Dalton *et al.*, 1968).



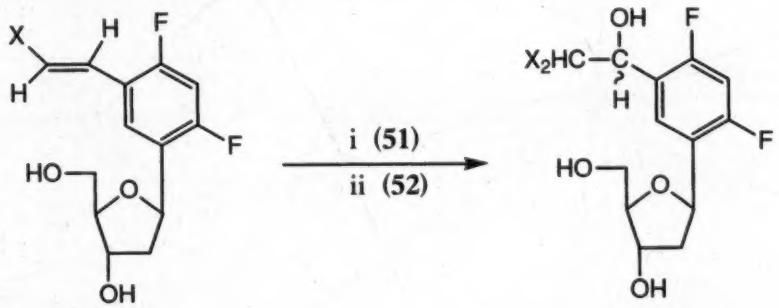
Scheme 17. Synthesis of 5-substituted-2,4-difluorophenyl pyrimidine nucleoside mimics **45-48**.

Reagents and conditions: i, *N*-bromosuccinimide (NBS), dioxane-water (3:7, v/v), HOAc, 25°C; ii, I_2, KIO_3, MeCN, 5N H_2SO_4, 60°C; iii, ICl, MeOH, 50°C; iv, NaOH, dioxane-water (3:7, v/v), 25°C.

In an earlier study, Knaus *et al.* (1989) showed that the reaction of 5-(2-halo-1-hydroxyethyl)-2'-deoxyuridines with H_2SO_4 in MeOH afforded the corresponding 5-(2-halo-1-methoxyethyl) derivatives (X = Br, Cl). Although it was expected that a similar treatment of compounds **45** and **46** with methanolic sulfuric acid would produce the corresponding 5-(2-halo-1-methoxyethyl) derivatives, no reaction was observed at either 25°C, or 50°C, for reaction times up to 72 hours as indicated by analytical TLC, or ^{1}H NMR, analysis. The 5-(2-iodoethyl-1-methoxyethyl) compound (**47**) was therefore prepared (56% yield) via a one-step reaction of the 5-vinyl compound (**44**) with iodine monochloride in MeOH (MeOI), as illustrated in Scheme 17. Compound **47** exhibited dual ^{1}H NMR resonances for the O*Me* group at δ 3.26 and 3.27, indicating that it consists of a mixture of two diastereomers that could not be separated by TLC or silica gel column chromatography. Treatment of the 5-(2-bromo-1-hydroxyethyl) (**45**), and 5-(1-hydroxy-2-iodoethyl) (**46**), compounds with NaOH in dioxane-water afforded the 5-(2-oxiranyl) derivative (**48**) in 71 and 81% yield, respectively (see Scheme 17).

The target 5-(2,2-dibromo-1-hydroxyethyl) compound (**51**) was synthesized by the regiospecific reaction of the (*E*)-5-(2-bromovinyl) compound (**49**) with *N*-bromosuccinimide in aqueous dioxane (85% yield). Alternatively, reaction of the (*E*)-5-(2-iodovinyl) compound (**50**) with iodine and iodic acid afforded the 5-(1-hydroxy-2,2-diiodoethyl) product (**52**, 90% yield) as illustrated in Scheme 18. The ^{1}H NMR and ^{13}C NMR spectra for the 5-(2,2-dibromo-1-hydroxyethyl) product (**51**) supported a regiospecific addition of HOBr to the (*E*)-5-(2-bromovinyl) substituent of **49**. For example, the ^{1}H NMR spectrum for **51** showed dual resonances at δ 6.02 and 6.05 (CHOHC*H*Br_2) and at δ 5.18 and 5.20 (C*H*OHCHBr_2), while the ^{13}C NMR spectrum

showed dual resonances at δ 73.46 and 73.21 (*C*HOHCHBr$_2$) and at δ 51.12 and 50.86 (CHOH*C*HBr$_2$). Products **51** and **52** exist as a mixture of two diastereomers (ratio 1:1), which differ in configuration (R and S) at the C-1 position of the 5-(2,2-dihalo-1-hydroxyethyl) moiety that could not be separated by silica gel column chromatography or multiple development TLC chromatography.



49, X = Br
50, X = I

51, X = Br
52, X = I

Scheme 18. Synthesis of 5-substituted-2,4-difluorophenyl pyrimidine nucleoside mimics **51** and **52**.

Reagents and conditions: i, *N*-bromosuccinimide (NBS), dioxane-water (3:7, v/v), HOAc, 25°C; ii, I_2, KIO_3, H_2O-MeCN, 5N H_2SO_4, 60°C.

Antiviral and anticancer activity evaluation

Replacement of the natural thymine base in thymidine by an unnatural aryl isostere provides a drug design strategy to develop a new class of 3rd generation *C*-aryl nucleoside mimics having desirable properties, which the natural nucleosides lack. Accordingly, *C*-aryl nucleoside mimics possess some potential advantages, compared to classical 5-substituted-2'-deoxyuridines, that include a) increased lipophilicity which could improve their ability to penetrate the blood-brain-barrier (BBB) to enhance their efficacy for the treatment of brain viral infections and brain tumors, b) *in vivo* resistance

to glycosidic bond cleavage by pyrimidine phosphorylases, and c) decreased host cell toxicity due to an inability to serve as substrates for thymidylate synthase (TS). A group of 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluorobenzenes having a variety C-5 two-carbon substituents [-$CH(OH)CH_2X$ (X = Br, I); -$CH(OMe)CH_2I$; oxiranyl; -$CH(OH)CHX_2$ (X = Br, I)] were investigated to probe the effect of steric and lipophilic parameters which are potential determinants of antiviral efficacy.

The 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-substituted-benzenes (**45-48, 51-52**) were evaluated for their antiviral activity in a wide variety of assay systems (De Clercq, 1994). Antiviral activities against herpes simplex virus type 1 (KOS), herpes simplex virus type 2 (G), thymidine kinase-deficient (TK^-) herpes simplex virus type 1 (strains KOS ACV^r, VMW-1837), vaccinia virus and vesicular stomatitis virus in E_6SM cells were determined. This group of compounds was inactive, as determined by reduction of virus-induced cytopathicity, in these antiviral assay systems at the highest concentration (μg/mL) tested [**45**, IC_{50} > 3.2; **46**, IC_{50} > 16; **47**, **48** and **51**, IC_{50} > 80; **52**, IC_{50} > 400]. In addition, the compounds did not reduce cytopathicity induced by parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie B4 virus or Punta Toro virus in Vero cell cultures (**45** and **47**, IC_{50} > 32 μg/mL; **46** and **48**, IC_{50} > 16 μg/mL; **51** and **52**, IC_{50} > 80 μg/mL), or respiratory syncytial virus in HeLa cell cultures [IC_{50} > 16 μg/mL (**45, 48**) and > 80 μg/mL (**46, 47, 51, 52**)].

Antiviral studies using varicella-zoster virus (VZV) infected human embryonic lung cells [TK^+ VZV (YS), TK^+ VZV (OKA), TK deficient VZV/TK^- (07/1) and TK deficient VZV/TK^- (YS/R)], showed that the inhibitory concentrations required to reduce virus plaque formation by 50% (IC_{50}, μM) for a viral input of 20 plaque forming units

(PFU) against these four respective strains of VZV were **45**, > 20, > 20 > 20, >20; **46**, 126, 100, > 50, >50; **7**, 141, 100, 130, 43; **48**, 159, 200, 121, 104; **51**, > 200, >200, >200, 126; and **52**, 85, 130, 115, > 50, respectively. The reference drug (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) showed IC_{50} values of 0.006 μM and 0.003 μM against VZV TK^+ YS and OKA strains, and > 150 μM against both VZV TK^- 07/1 and YS/R strains, respectively. These results indicate that expression of the viral TK enzyme, which would be expected to enhance phosphorylation to the 5'-monophosphate and 5'-diphosphate (by the accompanying thymidylate kinase activity), did not increase the activity of the test compounds against VZV.

Antiviral activity against cytomegalovirus (CMV), strains AD-169 and Davis, was determined using a cytopathicity (CPE) reduction assay (Snoeck *et al.*, 1991) in confluent human embryonic lung (HEL) cells. All compounds were inactive at the highest concentration tested (**45**, IC_{50} > 20 μM; **46-48** and **51-52**, IC_{50} > 200 μM).

This group of compounds was also evaluated for their activity against HIV-1 (strain III_B) and HIV-2 (strain ROD) in human T-lymphocytes (CEM cells). None of these compounds inhibited replication of HIV-1 or HIV-2 (**45**, IC_{50} > 10 μM; **46**, **48** and **52**, IC_{50} > 50 μM; **47** and **51**, IC_{50} > 250 μM).

The ability of these 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-substituted-benzenes (**45-48**, **51-52**) to inhibit the proliferation of murine leukemia cells (L1210/0), murine mammary carcinoma cells (FM3A/0), and human T-lymphocytes cells (Molt4/C8, CEM/O) in cell cultures by 50% (IC_{50} ± SEM) were evaluated using a previously reported procedure (De Clercq *et al.*, 1981). In these assays, this group of compounds exhibited modest inhibition of L1210/0 (IC_{50} = 24-233 μM range), FM3A/0

(IC_{50} = 46 to > 500 μM range), Molt4/C8 (IC_{50} = 37 to > 500 μM range), and CEM/0 (IC_{50} = 36-400 μM range) cell proliferation. The most potent inhibitor having a 5-(2-bromo-1-hydroxyethyl) substituent showed inhibitory values (IC_{50} ± SEM, μM) of 24.0 ± 8.1, 46.4 ± 6.9, 37.4 ± 2.0 and 36.7 ± 1.3 against these four cell lines, respectively.

There are a number of plausible reasons for the weak, or absence of, antiviral efficacy for this novel class of 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-substituted-benzenes. Thus, it is possible that the sugar moiety does not undergo phosphorylation by thymidine kinase (TK) to the 5'-monophosphate (5'-MP) or the compounds are good substrates for the enzyme but are not good binders. Support for this explanation is based on the observations that there are no distinct differences in antiviral activities between herpes simplex virus-1 (KOS), and either herpes simplex virus-1 TK^- KOS or VMW-1837 (TK^-/TK^+), or between TK^+ VZV and TK^- VZV, viral strains. Alternatively, the group of nucleoside mimics evaluated in this study may be devoid of antiviral activity due to the fact that, in their triphosphate form, they do not act as inhibitors of thymidylate synthase (TS). Evidence for this possibility is based on the fact that 5-fluoro-2'-deoxyuridine exhibits its anticancer effect primarily due to inhibition of DNA biosynthesis by blocking TS, the enzyme which catalyzes the methylation of 2'-deoxyuridine-5'-monophosphate to thymidine-5'-monophosphate (Langenbach *et al.*, 1972; Santi *et al.*, 1974). In addition, inhibition of TS is the mechanism by which certain nucleosides such as (*E*)-5-(2-iodovinyl)-2'-deoxyuridine and 5-(1-azidovinyl)-2'-deoxyuridine exhibit their cytostatic effect (Balzarini *et al.*, 1995).

Conclusions

The novel class of 1-(2-deoxy-β-D-ribofuranosyl)-7-substituted-isocarbostyrils reported here lacks anticancer/antiviral activity. The results obtained in this study suggest that these isocarbostyril mimics are not suitable thymidine mimics. In the event that the failure of this class of compounds to undergo phosphorylation to the 5'-monophosphate is responsible for the lack of anticancer/antiviral activities observed, it is possible that 5'-*cyclo*Sal-pronucleotide derivatives designed to adhere to the kinase-bypass concept (Meier *et al.*, 1999; Meier *et al.*, 1998) could result in the transformation of these inactive unnatural nucleoside mimics into a class of effective anticancer/antiviral agents.

In another effort, a group of nitric oxide donor 3'-*O*-nitro derivatives of 2'-deoxyuridine (**35**) and 2'-deoxycytidine (**38**), and 5'-*O*-nitro-2'-deoxyuridines (**40**), were designed to investigate the concept whether the concomitant release of cytotoxic nitric oxide may enhance the anticancer/antiviral efficacy of pyrimidine nucleosides. This group of compounds generally exhibits comparable and in most instances superior nitric oxide release, upon incubation with phosphate buffer containing 18 mM L-cysteine or in the presence of serum, compared to the reference drug isosorbide dinitrate. This group of 3'- and 5'-O-nitrate esters exhibit comparable *in vitro* cytotoxicity to 5-iodo-2'-deoxyuridine, but weaker cytotoxicity than 5-fluoro-2'-deoxyuridine, in a variety of cancer cell lines. These nitrate esters were devoid of antiviral activity except for 5-iodo-3'-O-nitro-2'-deoxyuridine, which showed marginal activity against HSV-1, HSV-2 and vaccinia virus in cell cultures.

Finally, a group of 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluorobenzenes (**45-48,51-52**) that possess C-5 2-halo-1-hydroxyethyl (X = Br, I), 2-iodo-1-methoxyethyl,

oxiranyl, and 2,2-dihalo-1-hydroxyethyl (X = Br, I) substituents were synthesized by the regiospecific addition of a halohydrin across a C-5 vinyl substituent for evaluation as antiviral and anticancer agents. This new class of "thymine replacement" analogs, in which the natural thymine moiety of deoxythymidine is replaced by an unnatural 5-substituted-2,4-difluorophenyl moiety designed to mimic thymine, exhibited low cytotoxicity but was devoid of antiviral activity in those viruses studied.

Above all, although the novel unnatural nucleosides reported in this thesis showed modest anticancer and/or antiviral activity, they could be potential anticancer/antiviral agents aginst other cancer cell lines and/or viruses that were not tested. In addition, these compounds could be used as potential radiopharmaceutical agents to image. The novel nitrooxy derivative nucleosides reported in this thesis showed potential NO release much more than classical drugs isosorbide dinitrate (ISDN) and glyceryl trinitrate (GTN). In view of chemistry, most of these compounds were synthesized in high yield by new synthetic procedures.

4. EXPERIMENTAL SECTION

General methods

Melting points were determined with a Thomas Hoover capillary apparatus and are uncorrected. ^{1}H NMR, ^{13}C NMR, and ^{19}F NMR spectra were measured on a Bruker AM-300 spectrometer in $CDCl_3$, MeOH-d_4, or DMSO-d_6. Proton chemical shifts (δ) are given relative to internal TMS (δ 0), and the assignment of exchangeable protons (N*H*, O*H*) was confirmed by addition of D_2O. The nuclear Overhauser enhancement (nOe) studies were performed under steady-state conditions using the Bruker NOE DIFF.AU software program (signal:noise ratio of 136 for a single pulse). DMSO-d_6 was dried using molecular sieves (type 3A, 1.6-mm pellets) and degassed by passage of dry argon gas at 22 °C just prior to use. Molecular tumbling time was not altered. ^{13}C NMR spectra were acquired using the *J* modulated spin echo technique where methyl and methine carbon resonances appear as positive peaks and methylene and quaternary carbons appear as negative peaks, and carbon chemical shifts (δ) are given relative to $CDCl_3$ (δ 77). Fluorine chemical shifts (δ) are given relative to external C_6F_6 (δ 0). Infrared spectra were recorded on a Nicolet Magna 550 IR spectrometer using air as reference. Elemental analyses were performed by the MicroAnalysis Service Laboratory, Department of Chemistry, University of Alberta. Silica gel 60A (Silicycle Co., 230-400 mesh) was used for all silica gel column flash chromatography separations. All NMR spectra were provided by Dr. V. Somayaji at the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. *In vitro* cell cytotoxicity (MTT assay) was performed according to previously established procedures (Alley *et al.*, 1988) by Mrs. W. Duan and Mrs. A. Zhou, at the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. *In*

vitro antiviral activity assays were performed following previously established procedures (De Clercq, 1994) by Dr. E. De Clercq and Dr. J. Balzarini at the Rega Institute for Medical Research, Leuven, Belgium. *In vitro* NO release were assayed using a modification of the previously reported procedures (Sako *et al.*, 1998) by Mrs. A. Zhou and Mrs. P. Khalili at the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. 3,5-Bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-α-D-ribofuranosyl chloride (**3**) (Wang *et al.*, 2001), (*E*)-1-trimethylsilyl-2- tributylstannylethene (**16**) (Cunico & Clayton, 1976), 5'-*O*-tosyl-2'-deoxyuridine (**39a**) (Henn *et al.*, 1993), 5'-O-tosyl-2'-deoxythymidine (**39b**) (Idziak *et al.*, 1993), 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-vinylbenzene (**44**), 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-(*E*)-5-(2-bromovinyl)benzene (**49**), and 1-(2'-deoxy-β-D-ribofuranosyl)-2,4-difluoro-(*E*)-5-(2-iodovinyl)benzene (**50**) (Wang *et al.*, 2001) were prepared according to the literature procedure. All other reagents were purchased from the Aldrich Chemical (Milwaukee, WI). The semiempirical PM3 calculation for isocarbosytril (**4**) was determined using the Alchemy 2000 simulation program, Version 2 (SciVision, Burlington, MA, U.S.A.).

1-[3,5-Bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl]isocarbostyril (5) and 1-[3,5-Bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-α-D-ribofuranosyl]isocarbostyril (6)

Dry MeCN (220 mL) and bis(trimethylsilyl)acetamide (17 mL, 68 mmol) were added to isocarbostyril (**4**, 10.0 g, 68 mmol) with stirring at 25 °C. Dissolution occurred within 10 min. Following a 30 min reaction time, additional MeCN (240 mL) and **3** (24.0 g, 56 mmol) were added. The reaction mixture was cooled to 0°C, $SnCl_4$ (13.8 mL of a 1 M solution in CH_2Cl_2, 13.8 mmol) was added dropwise, and the reaction was allowed to

proceed at 0 °C for 30 min with stirring. EtOAc (1000 mL) was added, and the resulting solution was consecutively extracted with saturated $NaHCO_3$ (3 × 200 mL) and then brine (200 mL). The organic extracts were dried (Na_2SO_4), the solvents were removed *in vacuo*, and the residue obtained was purified by flash silica gel column chromatography. Elution with CH_2Cl_2-EtOAc (40:0 → 40:1, v/v) gave the β-anomer **5** (10.1 g, 27%) as white crystals (hexane-CH_2Cl_2, mp 185-186 °C), and the α-anomer **6** (14.3 g, 39%) as white crystals (hexane-CH_2Cl_2, mp 172-173 °C), respectively.

β-Anomer 5: ^{1}H NMR ($CDCl_3$) δ 8.42 (d, J = 7.6 Hz, 1H, H-8), 7.95-8.04 (m, 4H, *ortho*-benzoyl hydrogens), 7.38-7.69 (complex multiplets, 8H, H-3, H-4, H-5, H-6, *meta*-benzoyl hydrogens), 6.83 (dd, J = 8.2, 5.8 Hz, 1H, H-1'), 6.48 (d, J = 7.6 Hz, 1H, H-7), 5.64 (br d, J = 6.7 Hz, 1H, H-3'), 4.68-4.70 (m, 2H, H-5'), 4.55-4.63 (m, 1H, H-4'), 2.90 (ddd, J = 14.3, 5.8, 1.6 Hz, 1H, H-2'α), 2.34 (ddd, J = 14.3, 8.2, 7.0 Hz, 1H, H-2'β); ^{13}C NMR ($CDCl_3$) δ 165.21, 165.09, 161.67, 140.15, 139.91, 136.59, 132.61, 131.13, 130.91, 128.90, 127.81, 127.59, 127.02, 125.90, 125.81, 125.14, 106.73, 85.51, 82.43, 75.47, 64.62, 38.54. Anal. calcd. for $C_{28}H_{21}Cl_2NO_6$: C, 62.46; H, 3.93; N, 2.60. Found: C, 62.56; H, 3.87; N, 2.64.

α-Anomer 6: ^{1}H NMR ($CDCl_3$) δ 8.43 (d, J = 8.2 Hz, 1H, H-3), 7.20-8.20 (complex multiplets, 12H, H-4, H-5, H-6, H-8, benzoyl hydrogens), 6.65 (dd, J = 6.7, 1.5 Hz, 1H, H-1'), 6.57 (d, J = 7.6 Hz, 1H, H-7), 5.63 (d, J = 6.1 Hz, 1H, H-3'), 4.93-4.99 (m, 1H, H-4'), 4.64 (dd, J = 11.9, 4.9 Hz, 1H, H-5'a), 4.56 (dd, J = 11.9, 5.2 Hz, 1H, H-5'b), 3.03 (ddd, J = 15.9, 6.7, 6.1 Hz, 1H, H-2'β), 2.69 (d, J = 15.9 Hz, H-2'α); ^{13}C NMR ($CDCl_3$) δ 165.14, 164.69, 161.63, 139.91, 139.85, 136.78, 132.45, 130.98, 130.89, 128.87, 128.61, 127.79, 127.49, 127.37, 126.80, 125.93, 125.71, 105.62, 88.03, 84.81, 75.23,

64.45, 38.89. Anal. calcd. for $C_{28}H_{21}Cl_2NO_6$: C, 62.46; H, 3.93; N, 2.60. Found C, 62.48; H, 3.89; N, 2.68.

1-(2-Deoxy-β-D-ribofuranosyl)isocarbostyril (7)

NaOMe (30 mg, 0.559 mmol) was added to a suspension of **5** (100 mg, 0.186 mmol) in MeOH (4 mL), and the mixture was stirred at 25 °C for 30 min. The reaction was quenched via addition of NH_4Cl (100 mg), and the solvent was removed *in vacuo* to give a residue that was purified via flash silica gel column chromatography using MeOH-CH_2Cl_2 (1:9, v/v) as eluent to afford **7** (43 mg, 90%) directly as white crystals: mp 109-110 °C; ^{1}H NMR (CD_3OD) δ 8.29 (d, J = 8.2 Hz, 1H, H-3), 7.77 (d, J = 7.6 Hz, 1H, H-8), 7.70 (dt, J = 7.9, 1.2 Hz, 1H, H-5), 7.61 (d, J = 7.9 Hz, 1H, H-6), 7.51 (ddd, J = 8.2, 7.9, 3.7 Hz, 1H, H-4), 6.72 (d, J = 7.6 Hz, 1H, H-7), 6.67-6.72 (m, 1H, H-1'), 4.38-4.46 (m, 1H, H-3'), 3.99 (dd, J = 7.3, 3.7 Hz, 1H, H-4'), 3.82 (dd, J = 11.9, 3.6 Hz, 1H, H-5'a), 3.75 (dd, J = 11.9, 3.9 Hz, 1-H, H-5'b), 2.41 (ddd, J = 13.4, 6.1, 3.4 Hz, 1H, H-2'α), 2.18 (ddd, J = 13.4, 7.0, 6.7 Hz, 1H, H-2'β); ^{13}C NMR (CD_3OD) δ 163.4, 138.4, 133.8, 128.1, 127.9, 127.7, 127.1, 126.4, 107.9, 88.8, 86.4, 72.3, 63.0, 41.9. Anal. calcd. for $C_{14}H_{15}NO_4$: C, 64.35; H, 5.78; N, 5.36. Found: C, 64.28; H, 5.81; N, 5.42.

1-(2-Deoxy-α-D-ribofuranosyl)isocarbostyril (8)

NaOMe (0.15 g, 2.79 mmol) was added to a suspension of **6** (0.50 g, 0.93 mmol) in MeOH (20 mL), and the reaction was allowed to proceed at 25 °C for 30 min with stirring. The reaction was quenched via addition of NH_4Cl (300 mg), and the solvent was removed *in vacuo* to give a residue that was purified by flash silica gel column

chromatography using MeOH-CH_2Cl_2 (1:9, v/v) as eluent to afford **8** (0.23 g, 94%) as a white foam: mp 86-88 °C; ^{1}H NMR (CD_3OD) δ 8.25 (d, J = 8.2 Hz, 1H, H-3), 7.69 (d, J = 7.6 Hz, 1H, H-8), 7.67 (ddd, J = 7.6, 7.0, 1.2 Hz, 1H, H-5), 7.59 (d, J = 7.6 Hz, 1H, H-6), 7.47 (ddd, J = 8.2, 7.0, 1.2 Hz, 1H, H-4), 6.70 (d, J = 7.8 Hz, 1H, H-7), 6.53 (dd, J = 7.3, 3.1 Hz, 1H, H-1'), 4.33-4.40 (m, 2H, H-3', H-4'), 3.68 (dd, J = 11.9, 4.3 Hz, 1H, H-5'a), 3.61 (dd, J = 11.9, 4.3 Hz, 1H, H-5'b), 2.82 (ddd, J = 14.3, 7.3, 7.1 Hz, 1H, H-2'β), 2.10 (ddd, J = 14.3, 3.1, 3.0 Hz, 1H, H-2'α); ^{13}C NMR (CD_3OD) δ 163.4, 138.6, 133.7, 128.2, 127.9, 127.8, 127.7, 127.1, 126.4, 107.3, 90.6, 88.4, 72.5, 63.4, 42.5. Anal. calcd. for $C_{14}H_{15}NO_4$: C, 64.35; H, 5.78; N, 5.36. Found C, 64.26; H, 5.73; N, 5.30.

1-[3,5-Bis-*O*-(*p*-chlorobenzoyl)-β-D-ribofuranosyl]-4,7-dinitroisocarbostyril (9)

Trifluoroacetic anhydride (10 mL) and ground NH_4NO_3 (0.45 g, 5.58 mmol) were added in aliquots to a solution of **5** (1.0 g, 1.86 mmol) in dry CH_2Cl_2 (10 mL), and the reaction was allowed to proceed at 25 °C for 1 h with stirring. The resultant yellow solution was poured onto cold water (100 mL), neutralized with $NaHCO_3$, and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic extracts were washed consecutively with water (2 x 100 mL), and then brine (2 x 50 mL), prior to drying (Na_2SO_4). The solvent was removed *in vauco*, and the residue was purified via flash silica gel column chromatography using hexane-EtOAc (5:1, v/v) as eluent to give the bis-3,5-*O*-ester (0.24 g, 21%) directly as pale yellow crystals: mp 170-171 °C; ^{1}H NMR ($CDCl_3$) δ 9.25 (d, J = 2.4 Hz, 1H, H-3), 9.09 (s, 1H, H-8), 8.85 (d, J = 9.2 Hz, 1H, H-6), 8.61 (dd, J = 9.2, 2.4 Hz, 1H, H-5), 7.88-8.04 (m, 4H, *ortho*-benzoyl hydrogens), 7.30-7.49 (m, 4H, *meta*-benzoyl hydrogens), 6.59 (dd, J = 7.6, 5.8 Hz, 1H, H-1'), 5.69 (d, J = 6.7 Hz, 1H, H-3'),

4.83-4.89 (m, 3H, H-4', H-5'), 3.15 (ddd, J = 13.4, 5.8, 1.2 Hz, 1H, H-2'α), 2.41 (ddd, J = 13.4, 7.6, 7.0 Hz, 1H, H-2'β); ^{13}C NMR ($CDCl_3$) δ 165.04, 165.01, 159.6, 146.8, 140.4, 140.0, 134.4, 133.1, 131.1, 130.7, 129.0, 128.8, 128.2, 127.4, 127.2, 125.6, 124.7, 124.3, 87.6, 84.0, 75.1, 64.1, 39.6. NaOMe (39 mg, 0.72 mmol) was added to a suspension of the bis-3,5-*O*-ester obtained above (0.14 g, 0.24 mmol) in MeOH (5.5 mL), and the reaction was allowed to proceed with stirring at 25 °C for 30 min. The reaction was quenched via addition of NH_4Cl (0.15 g), the solvent was removed *in vacuo*, and the residue obtained was purified via flash silica gel column chromatography using MeOH-CH_2Cl_2 (1:9, v/v) as eluent to afford **9** (70 mg, 89.5%) directly as light yellow crystals: mp 175-176 °C; ^{1}H NMR (DMSO-d_6) δ 9.57 (s, 1H, H-8), 8.93 (d, J = 2.4 Hz, 1H, H-3), 8.75 (d, J = 9.1 Hz, 1H, H-6), 8.66 (dd, J = 9.1, J = 2.4 Hz, 1H, H-5), 6.37 (t, J = 5.9 Hz, 1H, H-1'), 5.36 (d, J = 4.3 Hz, 1H, 3'-O*H*), 5.27 (t, J = 4.5 Hz, 1H, 5'-O*H*), 4.28-4.38 (m, 1H, H-3'), 3.96-4.03 (m, 1H, H-4'), 3.60-3.72 (m, 2H, H-5'a, H-5'b), 2.40-2.50 (m, 1H, H-2'α), 2.27 (ddd, J = 12.0, 6.1, 5.9 Hz, 1H, H-2'β); ^{13}C NMR (DMSO-d_6) δ 159.4, 145.8, 137.3, 133.0, 127.9, 127.7, 125.0, 124.0, 123.0, 88.3, 86.8, 69.5, 60.3, 41.0. Anal. calcd. for $C_{14}H_{13}N_3O_8$: C, 47.87; H, 3.73; N, 11.96. Found: C, 47.84; H, 3.63; N, 11.82.

1-[3,5-Bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl]-7-iodoisocarbostyril (10)

A solution of ICl (3.04 g, 18.4 mmol) in CH_2Cl_2 (30 mL) was added dropwise to a solution of **5** (8.0 g, 14.88 mmol) in dry CH_2Cl_2 (96 mL) with stirring, the reaction mixture was heated to reflux temperature, and then cooled to 25 °C. Saturated $NaHCO_3$ (200 mL) was added followed by the drop wise addition of saturated $Na_2S_2O_3$ until a clear solution was maintained. Extraction with CH_2Cl_2 (500 mL), washing the aqueous

fraction with CH_2Cl_2 (2 × 200 mL), drying the combined CH_2Cl_2 extracts (Na_2SO_4), and removal of the solvent *in vacuo* gave a residue that was purified by flash silica gel column chromatography. Elution with hexane-EtOAc (7:1, v/v) as eluent afforded a pale yellow oil (10.8 g) which was recrystallized from CH_2Cl_2-hexane to give **10** (8.64 g, 87 %) as white crystals: mp 145-146 °C; ^{1}H NMR ($CDCl_3$) δ 8.44 (dd, *J* = 7.9, 1.2 Hz, 1H, H-3), 8.01-8.05 (m, 4H, *ortho*-benzoyl hydrogens), 7.85 (s, 1H, H-8), 7.72-7.82 (m, 1H, H-5), 7.65 (dd, *J* = 7.6, 1.2 Hz, 1H, H-6), 7.57 (t, *J* = 7.9 Hz, 1H, H-4), 7.40-7.49 (m, 4H, *meta*-benzoyl hydrogens), 6.80 (dd, *J* = 8.5, 5.5 Hz, 1H, H-1'), 5.66 (d, J = 6.7 Hz, 1H, H-3'), 4.74-4.84 (m, 2H, H-5'), 4.60-4.66 (m, 1H, H-4'), 2.93 (ddd, *J* = 14.1, 5.5, 1.5 Hz, 1H, H-2'α), 2.36 (ddd, *J* = 14.1, 8.5, 6.7 Hz, 1H, H-2'β); ^{13}C NMR ($CDCl_3$) δ 165.1, 165.0, 160.8, 140.1, 139.8, 136.6, 133.5, 131.6, 130.9, 128.9, 127.9, 127.6, 127.4, 126.0, 123.3, 85.5, 82.8, 75.4, 72.6, 64.4, 38.8. Anal. calcd. for $C_{28}H_{20}Cl_2INO_6$: C, 50.62; H, 3.03; N, 2.10. Found: C, 50.42; H, 3.00; N, 2.14.

1-(2-Deoxy-β-D-ribofuranosyl)-7-iodoisocarbostyril (11)

NaOMe (488 mg, 9.04 mmol) was added to a suspension of **10** (2.0 g, 3.01 mmol) in MeOH (68 mL), and reaction was allowed to proceed at 25 °C for 30 min with stirring. The reaction was quenched via addition of NH_4Cl (1.5 g), the solvent was removed *in vacuo*, and the residue obtained was purified via flash silica gel column chromatography using MeOH- CH_2Cl_2 (1:9, v/v) as eluent to yield **11** (0.95 g, 82%) directly as white crystals: mp 158-159 °C; ^{1}H NMR (CD_3OD) δ 8.30 (s, 1H, H-8), 8.25 (d, *J* = 7.9 Hz, 1H, H-3), 7.70 (dd, *J* = 7.9, 7.0 Hz, 1H, H-5), 7.69 (d, *J* = 7.9 Hz, 1H, H-6), 7.58 (dd, *J* = 7.9, 7.0 Hz, 1H, H-4), 6.62 (dd, *J* = 6.7, 6.4 Hz, 1H, H-1'), 4.40-4.48 (m, 1H, H-3'), 3.98-4.05

(m, 1H, H-4'), 3.85 (dd, J = 11.9, 3.3 Hz, 1H, H-5'a), 3.76 (dd, J = 11.9, 3.7 Hz, 1H, H-5'b), 2.40-2.50 (complex m, 1H, H-2'α), 2.20 (ddd, J = 13.7, 6.7, 6.4 Hz, 1H, H-2'β); ^{13}C NMR (CD_3OD) δ 162.6, 138.4, 134.7, 131.5, 128.9, 128.8, 128.7, 124.2, 89.0, 86.6, 72.8, 72.0, 62.6, 42.3. Anal. calcd. for $C_{14}H_{14}INO_4$: C, 43.43; H, 3.64; N, 3.61. Found: C, 43.32; H, 3.69; N, 3.72.

1-(3,5-Bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl]-7-trifluoromethylisocarbostyril (12)

A mixture of **10** (1.0 g, 1.51 mmol), CuI (0.43 g, 2.26 mmol), and anhydrous KF (0.13 g, 2.26 mmol) in dry DMF (5 mL) was stirred under argon as the temperature was increased. When the reaction temperature reached 60 °C, $ClCF_2CO_2Me$ (0.32 mL, 3.01 mmol) was added, and the reaction was allowed to proceed at 120 °C for 6 h under an argon atmosphere. After cooling to 25 °C, the reaction mixture was poured onto ice-water (100 mL), the solution was filtered, and the residue was washed with CH_2Cl_2 (3 x 50 mL) and the aqueous layer was extracted with CH_2Cl_2 (3 × 100 mL). The combined CH_2Cl_2 extracts were washed with water (100 mL), the organic fraction was dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue obtained was purified via flash silica gel column chromatography using hexane-EtOAc (9:1, v/v) as eluent to give **12** (0.65 g, 71%) as a white foam: mp 85-86 °C; ^{1}H NMR ($CDCl_3$) δ 8.46 (d, J = 7.9 Hz, 1H, H-3), 7.90-8.04 (m, 4H, *ortho*-benzoyl hydrogens), 7.96 (s, 1H, H-8), 7.72-7.84 (m, 2H, H-5, H-6), 7.62 (ddd, J = 8.2, 7.9, 4.0 Hz, 1H, H-4), 7.36-7.50 (m, 4H, *meta*-benzoyl hydrogens), 6.71 (dd, J = 8.2, 5.5 Hz, 1H, H-1'), 5.63 (d, J = 6.4 Hz, 1H, H-3'), 4.73-7.85

(m, 2H, H-5'), 4.64-4.70 (m, 1H, H-4'), 2.99 (dd, J = 14.6, 5.5 Hz, 1H, H-2'α), 2.32 (ddd, J = 14.6, 8.2, 6.7 Hz, 1H, H-2'β); ^{19}F NMR ($CDCl_3$) δ 100.79 (s, CF_3).

1-(2-Deoxy-β-D-ribofuranosyl)-7-trifluoromethylisocarbostyril (13)

A solution of NaOMe in methanol (6 mL of 0.4 M) was added to **12** (0.60 g, 1 mmol), and the reaction was allowed to proceed with stirring at 25 °C for 30 min. The reaction was quenched via addition of NH_4Cl (0.5 g), the solvent was removed *in vacuo*, and the residue was purified via flash silica gel column chromatography using MeOH-CH_2Cl_2 (1:9, v/v) as eluent to give **13** (0.32 g, 97%) directly as white crystals: mp 163-164 °C; ^{1}H NMR (CD_3OD) δ 8.53 (s, 1H, H-8), 8.35 (d, J = 8.2 Hz, 1H, H-3), 7.74-7.83 (m, 2H, H-5, H-6), 7.59 (ddd, J = 8.2, 7.9, 3.2 Hz, 1H, H-4), 6.61 (t, J = 6.4 Hz, 1H, H-1'), 4.42-4.50 (m, 1H, H-3'), 4.01-4.08 (m, 1H, H-4'), 3.89 (dd, J = 11.9, 3.0 Hz, 1H, H-5'a), 3.77 (dd, J = 11.9, 3.3 Hz, 1H, H-5'b), 2.50 (ddd, J = 13.1, 6.4, 4.0 Hz, 1H, H-2'α), 2.25 (ddd, J = 13.1, 6.7, 6.4 Hz, 1H, H-2'β); ^{13}C NMR (CD_3OD) δ 162.8, 134.5, 132.8, 130.1 (q, J = 5.5 Hz, *C*-8), 129.9, 129.8, 126.5, 125.6 (q, J = 270.3 Hz, CF_3), 124.2, 107.7 (q, J = 31.8 Hz, *C*-7), 89.2, 87.1, 72.0, 62.4, 42.5; ^{19}F NMR (CD_3OD) δ 103.59 (s, CF_3). Anal. calcd. for $C_{15}H_{14}F_3NO_4$: C, 54.71; H, 4.28; N, 4.25. Found: C, 54.74; H, 4.20; N, 4.14.

1-[3,5-Bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl]-7-cyanoisocarbostyril (14)

Compound **10** (1.0 g, 1.51 mmol) was added to $Zn(CN)_2$ (107 mg, 0.91 mmol) and $(Ph_3P)_4Pd$ (70 mg, 0.06 mmol) in dry DMF (2 mL), and the yellow slurry was heated

at 80-90 °C under an argon atmosphere with stirring for 6 h. The reaction mixture was cooled to 25 °C, toluene (100 mL) was added, the mixture was washed with 2N NH_4OH (2 × 50 mL) and then brine (50 mL), and the organic fraction was dried (Na_2SO_4). Removal of the solvent *in vacuo* gave a residue that was purified via flash silica gel column chromatography using hexane-EtOAc (6:1, v/v) as eluent to give **14** (0.71 g, 84%) as white crystals (hexane-CH_2Cl_2): mp 185-186 °C; IR (KBr): 2221 (CN), 1726, 1669 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.34 (d, J = 7.9 Hz, 1H, H-3), 8.04 (s, 1H, H-8), 7.87-7.95 (m, 4H, *ortho*-benzoyl hydrogens), 7.68-7.78 (m, 2H, H-5, H-6), 7.55 (ddd, J = 7.9, 5.5, 2.7 Hz, 1H, H-4), 7.31-7.42 (m, 4H, *meta*-benzoyl hydrogens), 6.54 (dd, J = 7.9, 5.5 Hz, 1H, H-1'), 5.55 (dd, J = 6.4, 1.5 Hz, 1H, H-3'), 4.64-4.81 (m, 2H, H-5'), 4.54-4.62 (m, 1H, H-4'), 2.94 (ddd, J = 13.1, 5.5, 1.5 Hz, 1H, H-2'α), 2.17 (ddd, J = 13.1, 7.9, 6.7 Hz, 1H, H-2'β); ^{13}C NMR ($CDCl_3$) δ 165.1, 164.9, 160.4, 140.2, 140.0, 134.6, 133.9, 132.9, 131.0, 130.8, 130.7, 129.0, 128.8, 128.6, 128.1, 127.4, 127.3, 124.8, 124.0, 123.9, 115.3, 91.8, 86.5, 83.3, 75.1, 64.0, 39.1.

1-(2-Deoxy-β-D-ribofuranosyl)-7-cyanoisocarbostyril (15)

NaOMe (75 mg, 1.38 mmol) was added to a suspension of **14** (0.26 g, 0.46 mmol) in MeOH (8 mL), and the mixture was stirred at 25 °C for 30 min. The reaction was quenched via addition of NH_4Cl (0.3 g) and the solvent was removed *in vacuo* to give a residue that was purified via flash silica gel column chromatography using MeOH-CH_2Cl_2 (1:19, v/v) as eluent to afford **15** (0.13 g, 98%) directly as white crystals: mp 140-141 °C; IR (KBr): 2232 (CN), 1722 (C=O) cm^{-1}; ^1H NMR (CD_3OD) δ 8.65 (s, 1H, H-8), 8.28 (dd, J = 7.9, 1.2 Hz, 1H, H-3), 7.86 (ddd, J = 8.2, 7.9, 1.5 Hz, 1H, H-5), 7.75

(dd, J = 7.9, 1.2 Hz, 1H, H-6), 7.60 (ddd, J = 8.2, 7.9, 1.2 Hz, 1H, H-4), 6.51 (dd, J = 6.4, 6.1 Hz, 1H, H-1'), 4.42-4.48 (m, 1H, H-3'), 4.18-4.25 (m, 1H, H-4'), 3.88 (dd, J= 12.2, 3.0 Hz, 1H, H-5'a), 3.77 (dd, J = 12.2, 3.3 Hz, 1H, H-5'b), 2.52 (ddd, J = 13.7, 6.1, 3.7 Hz, 1H, H-2'α), 2.20 (dt, J = 13.7, 6.4 Hz, 1H, H-2'β); ^{13}C NMR (CD_3OD) δ 162.1, 138.2, 135.0, 134.6, 129.4, 128.7, 125.9, 124.5, 116.7, 91.8, 89.3, 87.4, 71.7, 62.3, 42.6. Anal. calcd. for $C_{15}H_{14}N_2O_4$: C, 62.93; H, 4.92; N, 9.78. Found: C, 62.87; H, 4.85; N, 9.53.

1-(2-Deoxy-β-D-ribofuranosyl)-7-(*E*)-(2-trimethylsilylvinyl)isocarbostyril (17)

$(Ph_3P)_2PdCl_2$ (102 mg, 0.145 mmol) was added to a mixture of **11** (0.56 g, 1.45 mmol) and (*E*)-1-trimethylsilyl-2-tributylstannylethene (1.78 g, 3 mmol) in dry MeCN (22 mL) under argon, and the mixture was stirred vigorously at 60 °C for 24 h. Removal of the solvent *in vacuo* gave a residue that was purified via flash silica gel column chromatography using hexane-EtOAc (1:3, v/v) as eluent to give **17** (0.43 g, 83%) as white crystals (hexane-CH_2Cl_2): mp 132-133 °C; ^{1}H NMR ($CDCl_3$) δ 8.41 (d, J = 8.2 Hz, 1H, H-3), 7.76 (d, J = 7.6 Hz, 1H, H-6), 7.67 (dd, J = 8.5, 6.7 Hz, 1H, H-5), 7.57, (s, 1H, H-8), 7.48 (dd, J = 8.2, 6.7 Hz, 1H, H-4), 7.10 (d, J = 18.9 Hz, 1H, C*H*=CHTMS), 6.54 (dd, J = 7.0, 6.4 Hz, 1H, H-1'), 6.32 (d, J = 18.9 Hz, 1H, CH=C*H*TMS), 4.65-4.72 (m, 1H, H-3'), 4.16-4.22 (m, 1H, H-4'), 4.02-4.10 and 3.65-3.74 (two m, 1H each, 3'-O*H*, 5'-O*H*), 3.88-4.00 (m, 2H, H-5'), 2.47-2.58 (m, 2H, H-2'), 0.18 (s, 9H, Si*Me*$_3$); ^{13}C NMR ($CDCl_3$) δ 161.7, 136.9, 135.2, 132.6, 132.1, 128.2, 126.9, 125.2, 124.9, 122.5, 117.9, 87.9, 87.3, 71.7, 62.6, 40.4, -1.1.

1-(2-Deoxy-β-D-ribofuranosyl)-7-(*E*)-(2-iodovinyl)isocarbostyril (18)

After dissolution of **17** (0.25 g, 0.7 mmol) in dry MeCN (12 mL), ICl (114 mg, 0.7 mmol) was added immediately, and the reaction was allowed to proceed at 0 °C for 15 min with stirring. Removal of the solvent *in vauco* gave a residue that was purified by flash silica gel column chromatography using hexane-EtOAc (1:3, v/v) as eluent to give a pale yellow oil which was recrystallized from CH_2Cl_2-hexane to yield **18** (0.20 g, 70%) directly as white crystals: mp 125-126 °C; ^{1}H NMR (CD_3OD) δ 8.35 (d, J = 8.2 Hz, 1H, H-3), 8.01 (s, 1H, H-8), 7.72-7.85 (m, 2H, H-5, H-6), 7.71 (d, J = 14.7 Hz, 1H, C*H*=CHI), 7.52-7.62 (m, 1H, H-4), 6.93 (d, J = 14.7 Hz, 1H, CH=C*H*I), 6.66 (dd, J = 6.7, 6.4 Hz, 1H, H-1'), 4.42-4.50 (m, 1H, H-3'), 3.97-4.02 (m, 1H, H-4'), 3.86 (dd, J = 11.9, 3.0 Hz, 1H, H-5'a), 3.77 (dd, J = 11.9, 3.7 Hz, 1H, H-5'b), 2.44 (ddd, J = 13.4, 6.7, 6.2 Hz, 1H, H-2'α), 2.24 (ddd, J = 13.4, 6.7, 6.2 Hz, 1H, H-2'β); ^{13}C NMR (CD_3OD + DMSO-d_6) δ 162.5, 139.9, 135.7, 134.1, 128.8, 127.9, 126.1, 126.0, 124.1, 117.8, 89.1, 86.5, 78.7, 71.8, 63.2, 42.3. Anal. calcd. for $C_{16}H_{16}INO_4$: C, 46.50; H, 3.90; N, 3.38. Found: C, 46.46; H, 3.75; N, 3.15.

1-[3,5-Bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl)]-7-(2-trimethylsilylethynyl)isocarbostyril (19)

A mixture of **10** (2.0 g, 3.01 mmol), CuI (90 mg), $(Ph_3P)_2PdCl_2$ (110 mg), and (trimethylsilyl)acetylene (0.85 mL, 6.02 mmol) in dry Et_3N (180 mL) was stirred at 50-60 °C for 5 h. The solvent was removed *in vacuo,* the residue was dissolved in CH_2Cl_2, washed with saturated aqueous ethylenediaminetetraacetic acid disodium salt and then brine, the organic fraction was dried (Na_2SO_4), and the solvent was removed *in vacuo*.

The residue obtained was purified via flash silica gel column chromatography using hexane-EtOAc (7:1, v/v) as eluent to give **19** (1.5 g, 78%) as white crystals (hexane-CH_2Cl_2): mp 102-103 °C; ^{1}H NMR ($CDCl_3$) δ 8.44 (d, J = 7.9 Hz, 1H, H-3), 8.00-8.05 (m, 4H, *ortho*-benzoyl hydrogens), 7.94 (d, J = 7.6 Hz, 1H, H-6), 7.82 (s, 1H, H-8), 7.76 (dd, J = 7.6, 7.0 Hz, 1H, H-5), 7.60 (dd, J = 7.9, 7.0 Hz, 1H, H-4), 7.41-7.49 (m, 4H, *meta*-benzoyl hydrogens), 6.80 (dd, J = 8.2, 5.5 Hz, 1H, H-1'), 5.64 (br d, J = 6.7 Hz, 1H, H-3'), 4.85 (dd, J = 12.2, 3.7 Hz, 1H, H-5'a), 4.72 (dd, J = 12.2, 3.4 Hz, 1H, H-5'b), 4.60-4.65 (m, 1H, H-4'), 2.94 (dd, J = 14.4, 5.5 Hz, 1H, H-2'α), 2.32 (ddd, J = 14.4, 8.2, 7.9 Hz, 1H, H-2'β), 0.26 (s, 9H, SiMe_3); ^{13}C NMR ($CDCl_3$) δ 165.1, 164.9, 160.7, 140.4, 140.0, 139.7, 135.5, 133.0, 131.0, 130.8, 130.0, 128.86, 128.82, 127.7, 127.5, 124.9, 122.1, 102.1, 98.9, 98.3, 85.6, 82.7, 75.4, 64.5, 38.7, 0.01.

1-(2-Deoxy-β-D-ribofuranosyl)-7-ethynylisocarbostyril (20)

A solution of NaOMe in methanol (18.5 mL of 0.2 N) was added to **19** (0.50 g, 0.79 mmol), and the reaction was allowed to proceed with stirring at 25 °C for 20 min. The reaction was quenched via addition of NH_4Cl (0.5 g) and the solvent was removed *in vacuo*. Purification of the residue via flash silica gel column chromatography using MeOH-CH_2Cl_2 (1:19, v/v) as eluent afforded **20** (0.22 g, 98%) directly as white crystals: mp 170-171 °C; ^{1}H NMR (CD_3OD) δ 8.30 (d, J = 8.2 Hz, 1H, H-3), 8.15 (s, 1H, H-8), 7.94 (d, J = 7.9 Hz, 1H, H-6), 7.76 (ddd, J = 8.5, 7.9, 1.5 Hz, 1H, H-5), 7.54 (ddd, J = 8.5, 8.2, 0.9 Hz, 1H, H-4), 6.62 (dd, J = 6.7, 6.4 Hz, 1H, H-1'), 4.39-4.47 (m, 1H, H-3'), 3.98-4.02 (m, 1H, H-4'), 3.84 (dd, J = 12.2, 3.4 Hz, 1H, H-5'a), 3.76 (dd, J= 12.2, 3.7 Hz, 1H, H-5'b), 3.73 (s, 1H, *H*-C≡C), 2.41 (ddd, J = 13.7, 6.4, 3.7 Hz, 1H, H-2'α), 2.21

(ddd, J = 13.7, 6.7, 6.7 Hz, 1H, H-2'β); ^{13}C NMR (CD_3OD) δ 165.0, 139.7, 136.8, 135.9, 131.1, 131.0, 128.4, 128.3, 104.6, 91.6, 89.3, 84.9, 74.7, 65.3, 44.8. Anal. calcd. for $C_{16}H_{15}NO_4$: C, 67.35; H, 5.29; N, 4.90. Found: C, 67.30; H, 5.32; N, 4.91.

1-[3,5-Bis-*O*- (*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl]-7-iodoethynylisocarbostyril (21)

N-Iodosuccinimide (214 mg, 0.95 mmol) and ground $AgNO_3$ (9 mg, 0.053 mmol) were added to a solution of **19** (0.50 g, 0.79 mmol) in dry DMF (5 mL) under argon at 0 °C with stirring. The reaction vessel was wrapped with aluminium foil to protect from light, and the reaction mixture was stirred at 0 °C for 2 h, and then at 25 °C for 1 h. The mixture was cooled to 0 °C, mixed with cold water (100 mL), and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic fractions were washed with water and then brine, dried (Na_2SO_4), the solvent was removed *in vacuo*, and the residue was purified via flash silica gel column chromatography using hexane-EtOAc (6:1, v/v) as eluent to give **21** (0.48 g, 88%) as white crystals (hexane-CH_2Cl_2): mp 165-166 °C; ^{1}H NMR ($CDCl_3$) δ 8.40 (d, J = 7.7 Hz, 1H, H-3), 7.94 (s, 1H, H-8), 7.85-7.95 (m, 4H, *ortho*-benzoyl hydrogens), 7.49-7.72 (m, 2H, H-5, H-6), 7.42-7.49 (m, 4H, *meta*-benzoyl hydrogens), 7.30-7.40 (m, 1H, H-4), 6.68-7.70 (m, 1H, H-1'), 5.52-5.61 (m, 1H, H-3'), 4.48-4.82 (m, 3H, H-5', H-4'), 2.80-3.02 (m, 1H, H-2'α), 2.20-2.35 (m, 1H, H-2'β).

1-(2-Deoxy-β-D-ribofuranosyl)-7-iodoethynylisocarbostyril (23)

A solution of NaOMe in MeOH (2.2 mL of 0.4 N) was added to **21** (0.25 g, 0.36 mmol) and the reaction was allowed to proceed at 25 °C for 15 min. The reaction was

quenched via addition of NH_4Cl (0.30 g), the solvent was removed *in vacuo*, and the residue was purified by flash silica gel column chromatography using MeOH-CH_2Cl_2 (1:19, v/v) as eluent to give **23** (0.14 g, 94%) directly as white crystals: mp 157-158 °C; ^{1}H NMR (CD_3OD) δ 8.29 (d, J = 8.2 Hz, 1H, H-3), 8.07 (s, 1H, H-8), 7.84 (d, J = 7.6 Hz, 1H, H-6), 7.73 (ddd, J = 8.2, 7.9, 1.2 Hz, 1H, H-5), 7.54 (ddd, J = 8.2, 7.9, 1.2 Hz, 1H, H-4), 6.60 (dd, J = 6.7, 6.4 Hz, 1H, H-1'), 4.35-4.46 (m, 1H, H-3'), 3.98-4.01 (m, 1H, H-4'), 3.84 (dd, J = 12.2, 3.4 Hz, 1H, H-5'a), 3.76 (dd, J = 12.2, 3.9 Hz, 1H, H-5'b), 2.45 (ddd, J = 13.4, 6.4, 3.4 Hz, 1H, H-2'α), 2.20 (ddd, J = 13.4, 7.0, 6.7 Hz, 1H, H-2'β); ^{13}C NMR (CD_3OD) δ 162.3, 137.3, 134.3, 133.5, 128.6, 128.5, 125.7, 103.6, 89.0, 88.4, 86.7, 72.1, 62.7, 42.3, 14.1. Anal. calcd. for $C_{16}H_{14}INO_4$: C, 46.73; H, 3.43; N, 3.40. Found: C, 46.87; H, 3.39; N, 3.44.

1-[3,5-Bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl]-7-(2-bromoethynyl)isocarbostyril (22)

N-Bromosuccinimide (0.16 g, 0.9 mmol) and ground $AgNO_3$ (9 mg, 0.053 mmol) were added to a solution of **19** (0.45 g, 0.71 mmol) in dry DMF (5 mL) under argon at 0 °C. The reaction vessel was wrapped with aluminium foil to protect from light, and the reaction mixture was stirred at 0 °C for 2 h and then at 25 °C for 4 h. The reaction mixture was cooled to 0 °C, mixed with cold-water (100 mL), and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic extracts were washed with water and then brine, the organic fraction was dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue was purified by flash silica gel column chromatography using hexane-EtOAc (6:1, v/v) as

eluent to give **22** (0.34 g, 75%) as white crystals (hexane-CH_2Cl_2): mp 151-152 °C. Product **22** was hydrolzyed to **24** as indicted below.

1-(2-Deoxy-β-D-ribofuranosyl)-7-(2-bromoethynyl)isocarbostyril (24)

A solution of NaOMe in MeOH (1.6 mL of 0.4 N) was added to **22** (0.17 g, 0.265 mmol) with stirring and the reaction was allowed to proceed at 25 °C for 15 min. The reaction was quenched via addition of NH_4Cl (0.15 g) and the solvent was removed *in vacuo*. Purification of the residue by flash silica gel column chromatography using MeOH-CH_2Cl_2 (1:19, v/v) as eluent yielded **24** (87 mg, 90%) directly as white crystals: mp 127-128 °C; ^{1}H NMR (CD_3OD) δ 8.76 (s, 1H, H-8), 8.70 (d, J = 8.2 Hz, 1H, H-3), 8.27 (dd, J = 7.9, 1.2 Hz, 1H, H-6), 7.69 (ddd, J = 7.9, 7.0, 1.5 Hz, 1H, H-5), 7.48 (ddd, J = 8.2, 7.0, 0.9 Hz, 1H, H-4), 6.50 (dd, J = 6.4, 6.1 Hz, 1H, H-1'), 4.36-4.44 (m, 1H, H-3'), 3.97-4.05 (m, 1H, H-4'), 3.74-3.90 (m, 2H, H-5'), 2.47 (ddd, J = 13.7, 6.4, 4.0 Hz, 1H, H-2'α), 2.18 (ddd, J = 13.7, 6.7, 6.1 Hz, 1H, H-2'β); ^{13}C NMR (CD_3OD) δ 167.0, 136.4, 135.4, 134.3, 128.4, 128.1, 126.3, 126.2, 126.0, 108.1, 89.1, 87.2, 72.0, 62.7, 52.2, 42.4. Anal. calcd. for $C_{16}H_{14}BrNO_4$: C, 52.76; H, 3.87; N, 3.84. Found: C, 52.82; H, 3.76; N, 3.69.

1-[3,5-Bis-*O*-(*p*-chlorobenzoyl)-2-deoxy-β-D-ribofuranosyl]-7-propynylisocarbostyril (25)

Dry Et_3N (70 mL), $(Ph_3P)_2PdCl_2$ (53 mg, 0.075 mmol), and CuI (0.057 g, 0.3 mmol) were added to **10** (1.0 g, 1.51 mmol), the mixture was cooled to –78 °C, and propyne was added until a final volume of 93 mL was reached. The reaction vessel was

sealed, and the reaction was allowed to proceed at 25 °C with stirring for 5 h. Excess propyne was allowed to escape by venting the reaction vessel, and the solvent was removed *in vacuo*. The residue was dissolved in CH_2Cl_2 (200 mL) and consecutively extracted with 5% EDTA (2 × 50 mL), water (100 mL) and then brine (50 mL). The organic fraction was dried (Na_2SO_4), the solvents were removed *in vacuo*, and the residue was purified by flash silica gel column chromatography using hexane-EtOAc (7:1, v/v) as eluent to give **25** (0.59 g, 68%) as a white foam: mp 125-126 °C; ^{1}H NMR ($CDCl_3$) δ 8.42 (d, J = 7.9 Hz, 1H, H-3), 7.98-8.04 (m, 4H, *ortho*-benzoyl hydrogens), 7.92 (d, J = 7.6 Hz, 1H, H-6), 7.73 (ddd, J = 8.2, 7.6, 1.5 Hz, 1H, H-5), 7.66 (s, 1H, H-8), 7.54 (dd, J = 8.2, 7.9 Hz, 1H, H-4), 7.38-7.47 (m, 4H, *meta*-benzoyl hydrogens), 6.84 (dd, J = 8.2, 5.7 Hz, 1H, H-1'), 5.62-5.66 (m, 1H, H-3'), 4.79 (dd, J = 12.2, 3.7 Hz, 1H, H-5'a), 4.71 (dd, J = 12.2, 3.4 Hz, 1H, H-5'b), 4.56-4.62 (m, 1H, H-4'), 2.86 (ddd, J = 14.3, 5.7, 1.5 Hz, 1H, H-2'α), 2.34 (ddd, J = 14.3, 8.2, 7.0 Hz, 1H, H-2'β), 2.01 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 165.1, 164.9, 160.7, 140.0, 139.7, 136.0, 132.7, 131.0, 130.9, 128.8, 128.7, 127.7, 127.5, 127.3, 125.1, 125.0, 102.8, 89.0, 85.2, 82.5, 75.3, 64.5, 38.6, 4.2.

1-(2-Deoxy-β-D-ribofuranosyl)-7-propynylisocarbostyril (1)

A solution of NaOMe in MeOH (4.2 mL of 0.4 N) was added to **25** (0.4 g, 0.69 mmol) and the reaction was allowed to proceed at 25 °C for 15 min with stirring. The reaction was quenched via addition of NH_4Cl (0.3 g) and the solvent was removed *in vacuo* to give a residue that was purified by flash silica gel column chromatography using MeOH-CH_2Cl_2 (1:19, v/v) as eluent to afford **1** (186 mg, 90%) as a white foam: mp 145-146 °C; ^{1}H NMR (CD_3OD) δ 8.22-8.32 (m, 1H, H-3), 7.95 (s, 1H, H-8), 7.84 (d, J = 7.6

Hz, 1H, H-6), 7.65-7.76 (m, 1H, H-5), 7.52-7.60 (m, 1H, H-4), 6.63 (dd, *J* =7.5, 6.3 Hz, 1H, H-1'), 4.39-4.48 (m, 1H, H-3'), 3.98-4.04 (m, 1H, H-4'), 3.83 (dd, *J* = 12.1, 3.5 Hz, 1H, H-5'a), 3.76 (dd, *J* = 12.1, 3.7 Hz, 1H, H-5'b), 2.42 (ddd, *J* = 13.7, 6.2, 3.5 Hz, 1H, H-2'α), 2.17 (ddd, *J* = 13.7, 7.4, 6.5 Hz, 1H, H-2'β), 2.10 (s, 3H, C*H*$_3$); ^{13}C NMR (CD_3OD) δ 162.4, 138.4, 137.6, 134.7, 131.5, 128.8, 128.2, 126.0, 124.2, 103.9, 90.0, 89.0, 86.7, 72.1, 62.7, 42.1, 3.9. Anal calcd. for $C_{17}H_{17}NO_4$: C, 68.21; H, 5.72; N,4.67. Found: C, 68.43; H, 5.61; N, 4.52.

General Method for the Preparation of 5-Substituted-2,3'-anhydro-5'-*O*-benzoyl-2'-deoxyuridines (33a-d)

A solution containing either **32a, 32b, 32c** or **32d** (65.7 mmol) and triphenylphosphine (Ph_3P, 25.9 g, 98.6 mmol) in DMF (90 mL) was prepared, a solution comprised of diisopropyl azodicarboxylate (DIAD, 19.5 mL, 98.6 mmol) and benzoic acid (12.1 g, 98.6 mmol) in DMF (40 mL) was added drop wise with stirring at 25 °C, and the reaction was allowed to proceed for 30 min at 25 °C. An additional aliquot of Ph_3P (98.6 mmol) and DIAD (98.6 mmol) was added, the reaction was allowed to proceed for 1 h, Et_2O (400 mL) was added, and the mixture was stirred for 10 min at 25 °C. The resulting suspension was cooled to ice-bath temperature, the white crystalline precipitate was collected by filtration, and washed with cold Et_2O to give the respective product **33a, 33b, 33c** or **33d**. Physical and spectral data for **33a-d** are listed below.

2,3'-Anhydro-5'-*O*-benzoyl-2'-deoxyuridine (33a)

White solid; yield, 81%; mp 225-226 °C; ^{1}H NMR (DMSO-d_6): δ 7.93 (d, J = 7.8 Hz, 2H, *ortho*-benzoyl hydrogens), 7.66 (d, J = 7.5 Hz, 1H, H-6), 7.63-7.68 (m, 1H, *para*-benzoyl hydrogen), 7.47-7.52 (m, 2H, *meta*-benzoyl hydrogens), 5.97 (d, J = 3.3 Hz, 1H, H-1'), 5.78 (d, J = 7.5 Hz, 1H, H-5), 5.45 (br s, 1H, H-3'), 4.50-4.60 (m, 2H, H-5'), 4.37 (dd, J = 11.7, 6.3 Hz, 1H, H-4'), 2.52-2.88 (m, 2H, H-2'); ^{13}C NMR (DMSO-d_6): δ 170.05, 165.14, 153.50, 140.57, 133.34, 129.06, 128.92, 128.55, 107.88, 86.94, 81.86, 77.28, 62.48, 32.68. Anal. calcd. for $C_{16}H_{14}N_2O_5$: C, 61.14; H, 4.49; N, 8.91. Found: C, 61.08; H, 4.51; N, 8.80.

2,3'-Anhydro-5'-*O*-benzoyl-2'-deoxythymidine (33b)

White solid; yield, 74%; mp 245-246 °C; ^{1}H NMR (DMSO-d_6): δ 7.90 (dd, J = 6.9, 1.2 Hz, 2H, *ortho*-benzoyl hydrogens), 7.63-7.68 (m, 1H, *para*-benzoyl hydrogen), 7.57 (s, 1H, H-6) 7.46-7.57 (m, 2H, *meta*-benzoyl hydrogens), 5.90 (d, J = 3.6 Hz, 1H, H-1'), 5.42 (br s, 1H, H-3'), 4.50-4.61 (m, 2H, H-5'), 4.36 (dd, J = 11.7, 5.4 Hz, 1H, H-4'), 2.53-2.88 (m, 2H, H-2'), 1.72 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 170.63, 165.14, 153.13, 136.32, 133.30, 129.04, 128.70, 128.37, 116.00, 86.81, 81.80, 77.07, 62.30, 32.71, 12.85. Anal. calcd. for $C_{17}H_{16}N_2O_5$: C, 62.19; H, 4.91; N, 8.53. Found: C, 62.06; H, 4.95; N, 8.40.

5-Fluoro-2,3'-anhydro-5'-*O*-benzoyl-2'-deoxyuridine (33c)

White solid; yield, 90%; mp 235-236 °C; ^{1}H NMR (DMSO-d_6): δ 8.10 (d, J = 5.4 Hz, 1H, H-6), 7.89-7.91 (m, 2H, *ortho*-benzoyl hydrogens), 7.62-7.68 (m, 1H, *para*-

benzoyl hydrogen), 7.46-7.51 (m, 2H, *meta*-benzoyl hydrogens), 5.92 (d, J = 3.9 Hz, 1H, H-1'), 5.48 (br s, 1H, H-3'), 4.54-4.65 (m, 2H, H-5'), 4.43 (dd, J = 11.7, 6.3 Hz, 1H, H-4'), 2.67-2.71 (m, 1H, H-2'α), 2.56 (dt, J = 13.5, 2.7 Hz, 1H, H-2'β); ^{13}C NMR (DMSO-d_6): δ 165.11 (CO_2), 162.16 (d, J_{CCF} = 16.5 Hz, C-4 *C*O), 151.22 (*C*-2), 144.31 (d, J_{CF} = 248.3 Hz, *C*-5), 133.32, 128.99 and 128.51 (phenyl *C*H), 128.90 (phenyl *C*-1), 125.34 (d, J_{CCF} = 33.0 Hz, *C*-6), 87.45 (*C*-1'), 82.11 (*C*-3'), 77.70 (*C*-4'), 62.21 (*C*-5'), 32.46 (*C*-2'). Anal. calcd. for $C_{16}H_{13}FN_2O_5$: C, 57.83; H, 3.94; N, 8.43. Found: C, 57.61; H, 3.70; N, 8.76.

5-Iodo-2,3'-anhydro-5'-*O*-benzoyl-2'-deoxyuridine (33d)

White solid; yield, 82%; mp 224-225 °C; ^{1}H NMR (DMSO-d_6): δ 8.30 (s, 1H, H-6), 7.86-7.94 (m, 2H, *ortho*-benzoyl hydrogens), 7.60-7.67 (m, 1H, *para*-benzoyl hydrogen), 7.46-7.51 (m, 2H, *meta*-benzoyl hydrogens), 5.98 (d, J = 3.6 Hz, 1H, H-1'), 5.46 (br s, 1H, H-3'), 4.54-4.61 (m, 2H, H-5'), 4.42 (ddd, J = 11.7, 6.3, 5.4 Hz, 1H, H-4'), 2.55-2.88 (m, 2H, H-2'); ^{13}C NMR (DMSO-d_6): δ 166.57, 165.14, 153.80, 145.05, 133.34, 128.99, 128.88, 128.55, 87.15, 81.99, 80.68, 77.64, 62.16, 32.48. Anal. calcd. for $C_{16}H_{13}IN_2O_5$: C, 43.66; H, 2.98; N, 6.36. Found: C, 43.78; H, 3.10; N, 6.21.

General Method for the Preparation of 5-Substituted-3'-*O*-nitro-5'-O-benzoyl-2'-deoxyuridines (34a-d)

A mixture containing either **33a, 33b, 33c** or **33d** (26.1 mmol) and NH_4NO_3 (31.3 g, 391.5 mmol) in dry DMF (120 mL) was stirred at 110-120 °C for 12 h under argon. The solvent was removed in vacuo, and the residue was purified via silica gel flash

column chromatography using hexane-EtOAc (1:3, 1:2, 1:7 or 1:1, v / v, respectively) as eluent to give the corresponding product **34a, 34b, 34c** or **34d**. Physical and spectral data for **34a-d** are listed below.

3'-*O*-Nitro-5'-*O*-benzoyl-2'-deoxyuridine (34a)

White crystals (CH_2Cl_2-hexane); yield, 31%; mp 168-170 °C; ^{1}H NMR (DMSO-d_6): δ 11.43 (s, 1H, *NH*), 7.99 (d, J = 7.8 Hz, 2H, *ortho*-benzoyl hydrogens), 7.67 (d, J = 7.8 Hz, 1H, H-6), 7.65-7.70 (m, 1H, *para*-benzoyl hydrogen), 7.52-7.57 (m, 2H, *meta*-benzoyl hydrogens), 6.13 (dd, J = 7.8, 6.3 Hz, 1H, H-1'), 5.76 (dd, J = 5.4, 1.2 Hz, 1H, H-3'), 5.60 (d, J = 7.8 Hz, 1H, H-5), 4.48-4.61 (m, 3H, H-4', H-5'), 2.56-2.74 (m, 2H, H-2'); ^{13}C NMR (DMSO-d_6): δ 165.32, 162.77, 150.15, 140.44, 133.48, 129.15, 129.06, 128.72, 102.06, 84.78, 83.29, 79.17, 64.04, 34.24. Anal. calcd. for $C_{16}H_{15}N_3O_8$: C, 50.93; H, 4.01; N, 11.14. Found: C, 50.81; H, 4.22; N, 11.19.

3'-*O*-nitro-5'-*O*-benzoyl-2'-deoxythymidine (34b)

White crystals (CH_2Cl_2-hexane); yield, 34%; mp 109-110 °C; ^{1}H NMR (DMSO-d_6): δ 11.40 (s, 1H, *NH*), 8.02 (dd, J = 8.4, 1.5 Hz, 2H, *ortho*-benzoyl hydrogens), 7.66-7.71 (m, 1H, *para*-benzoyl hydrogen), 7.52-7.57 (m, 2H, *meta*-benzoyl hydrogens), 7.44 (s, 1H, H-6), 6.17 (dd, J = 8.4, 6.9 Hz, 1H, H-1'), 5.80 (br d, J = 6.6 Hz, 1H, H-3'), 4.62 (dt, J = 7.8, 2.1 Hz, 1H, H-4'), 4.49-4.57 (m, 2H, H-5'), 2.57-2.72 (m, 2H, H-2'), 1.59 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 165.30, 163.35, 150.16, 135.43, 133.49, 129.14, 129.06, 128.74, 109.89, 83.99, 83.32, 78.97, 64.03, 34.21, 11.79. Anal. calcd. for $C_{17}H_{17}N_3O_8$: C, 52.18; H, 4.38; N, 10.74. Found: C, 52.24; H, 4.45; N, 10.66.

5-Fluoro-3'-*O*-nitro-5'-*O*-benzoyl-2'-deoxyuridine (34c)

White crystals (EtOAc-hexane); yield, 50%; mp 168-170 °C; ^{1}H NMR (DMSO-d_6): δ 11.15 (br s, 1H, *NH*), 7.86 (dd, *J* = 8.4, 1.5 Hz, 2H, *ortho*-benzoyl hydrogens), 7.76 (d, *J* = 6.0 Hz, 1H, H-6), 7.63-7.68 (m, 1H, *para*-benzoyl hydrogen), 7.47-7.52 (m, 2H, *meta*-benzoyl hydrogen), 6.55 (d, *J* = 3.3 Hz, 1H, H-1'), 5.46 (br d, *J* = 1.5 Hz, 1H, H-3'), 4.52-4.62 (m, 2H, H-5'), 4.43 (dd, *J* = 11.7, 6.0 Hz, 1H, H-4'), 2.56-2.63 (m, 2H, H-2'); ^{13}C NMR (DMSO-d_6): δ 165.11 (*C*O_2), 153.34 (d, J_{CCF} = 26.4 Hz, C-4 *C*=O), 149.82 (d, *J* = 12.8 Hz, C-2 *C*=O), 145.28 (d, J_{CF} = 242.8 Hz, C-5), 136.47 (d, J_{CCF} = 25.3 Hz, C-6), 133.36, 129.02, 128.53 and 128.38 (phenyl carbons), 82.61 (C-1'), 78.92 (C-3'), 77.57 (C-4'), 62.16 (C-5'), 31.75 (C-2'). Anal. calcd. for $C_{16}H_{14}FN_3O_8$: C, 48.62; H, 3.57; N, 10.63. Found: C, 48.79; H, 3.46; N, 10.71.

5-Iodo-3'-*O*-nitro-5'-*O*-benzoyl-2'-deoxyuridine (34d)

METHOD A: White foam; yield, 2.8%; mp 85-86 °C; ^{1}H NMR ($CDCl_3$): δ 9.40 (s, 1H, *NH*), 8.02-8.11 (m, 2H, *ortho*-benzoyl hydrogens), 7.89 (s, 1H, H-6), 7.60-7.70 (m, 1H, *para*-benzoyl hydrogens), 7.43-7.52 (m, 2H, *meta*-benzoyl hydrogens), 6.21 (dd, *J* = 6.6, 5.7 Hz, 1H, H-1'), 5.64 (d, *J* = 6.6 Hz, 1H, H-3'), 4.68-4.76 (m, 2H, H-5'), 4.58 (br s, 1H, H-4'), 2.81 (dd, *J* = 14.4, 5.1 Hz, 1H, H-2'α), 2.37 (ddd, *J* = 14.7, 7.5, 7.2 Hz, 1H, H-2'β); ^{13}C NMR ($CDCl_3$): δ 165.87, 159.53, 149.47, 143.43, 133.84, 129.60, 128.87, 128.81, 85.92, 82.60, 81.52, 69.03, 63.91, 36.97. Anal. calcd. for $C_{16}H_{14}IN_3O_8$: C, 38.19; H, 2.80; N, 8.35. Found: C, 38.48, H, 3.15; N, 8.11.

METHOD B: A mixture of **34a** (1.0 g, 2.65 mmol) and NaN_3 (0.69 g, 10.6 mmol) in dry MeCN (50 mL) was cooled in an ice-bath, a solution of ICl (1.07 g, 6.6 mmol) in MeCN (5 mL) was added drop wise during 5 min, and the reaction was allowed to proceed at 25 °C for 48 h with stirring under an argon atmosphere. Removal of the solvent in vacuo gave a residue that was purified via flash silica gel column chromatography using hexane-EtOAc (1:1, v / v) as eluent to afford **34d** (1.1 g, 82.5%) as a white foam, which was identical (mp, ^{1}H NMR) to **34d** described above under Method A.

General Method for the Preparation of 5-Substituted-3'-*O*-nitro-2'-deoxyuridines (35a-d)

A solution of NaOMe in MeOH (12.75 mL of 0.5 M) was added to either **34a, 34b, 34c** or **34d** (3.18 mmol) and the reaction was allowed to proceed at 25 °C for 1 h with stirring. The reaction was quenched by addition of NH_4Cl (0.5 g) and the solvent was removed in vacuo. Purification of the residue by flash silica gel column chromatography using MeOH: CH_2Cl_2 (1:9, v/v) as eluent yielded the respective product **35a, 35b, 35c** or **35d**. Physical and spectral data for **35a-d** are listed below.

3'-*O*-Nitro-2'-deoxyuridine (35a)

Directly as white crystals; yield, 91%; mp 188-190 °C; ^{1}H NMR (DMSO-d_6): δ 11.35 (br s, 1H, N*H*), 7.88 (d, *J* = 8.4 Hz, 1H, H-6), 6.13 (dd, *J* = 8.7, 5.7 Hz, 1H, H-1'), 5.67 (d, *J* = 8.4 Hz, 1H, H-5), 5.58 (d, *J* = 6.0 Hz, 1H, H-3'), 5.25 (br s, 1H, 5'-*OH*), 4.21 (d, *J* = 1.8 Hz, 1H, H-4'), 3.60-3.74 (m, 2H, H-5'), 2.45-2.56 (m, 1H, H2'α), 2.40 (ddd, *J* = 14.7, 8.7, 6.3 Hz, 1H, H-2'β); ^{13}C NMR (DMSO-d_6): δ 162.86, 150.25, 140.12, 102.13,

84.59, 83.84, 82.46, 61.26, 35.23. Anal. calcd. for $C_9H_{11}N_3O_7$: C, 39.57; H, 4.06; N, 15.38. Found: C, 39.49; H, 3.95; N, 15.28.

3'-*O*-Nitro-2'-deoxythymidine (35b)

White crystals (CH_2Cl_2-hexane); yield, 92%; mp 137-139 °C; ^{1}H NMR (DMSO-d_6): δ 11.36 (s, 1H, N*H*), 7.72 (s, 1H, H-6), 6.14 (dd, *J* = 8.4, 6.0 Hz, 1H, H-1'), 5.59 (d, *J* = 5.7 Hz, 1H, H-3'), 5.31 (t, *J* = 5.4 Hz, 1H, 5'-O*H*), 4.18 (d, *J* = 1.8 Hz, 1H, H-4'), 3.67 (dd, *J* = 5.1, 3.3 Hz, 2H, H-5'), 2.37-2.52 (m, 2H, H-2'), 1.78 (s, 3H, C*H*$_3$); ^{13}C NMR (DMSO-d_6): δ 163.50 (C-4 *C*=O), 150.31 (C-2 *C*=O), 135.66 (C-6), 109.76 (C-5), 84.54 (C-1'), 83.45 (C-3'), 82.23 (C-4'), 61.28 (C-5'), 34.91 (C-2'), 12.24 (*C*H$_3$). Anal. calcd. for $C_{10}H_{13}N_3O_7$: C, 41.82; H, 4.56; N, 14.63. Found: C, 41.77; H, 4.51; N, 14.57.

5-Fluoro-3'-*O*-nitro-2'-deoxyuridine (35c)

Directly as white crystals; yield, 95%; mp 195-197 °C; ^{1}H NMR (DMSO-d_6): δ 11.20 (br s, 1H, N*H*), 7.76 (d, *J* = 6.0 Hz, 1H, H-6), 6.49 (d, *J* = 2.7 Hz, 1H, H-1'), 5.28 (d, *J* = 1.2 Hz, 1H, H-3'), 5.02 (br s, 1H, 5'-O*H*), 4.20 (dt, *J* = 6.3, 2.4 Hz, 1H, H-4'), 3.42-3.58 (m, 2H, H-5'), 2.45-2.56 (m, 2H, H-2'); ^{13}C NMR (DMSO-d_6): δ 153.31 (d, J_{CCF} = 26.4 Hz, C-4 *C*=O), 150.12 (C-2 *C*=O), 145.17 (d, J_{CF} = 241.7 Hz, C-5), 136.04 (d, J_{CCF} = 29.7 Hz, C-6), 85.85 (C-1'), 78.62 (C-3'), 77.26 (C-4'), 59.28 (C-5'), 31.68 (C-2'). Anal. calcd. for $C_9H_{10}FN_3O_7$: C, 37.12; H, 3.46; N, 14.43. Found: C, 36.98; H, 3.55; N, 14.54.

5-Iodo-3'-*O*-nitro-2'-deoxyuridine (35d)

Directly as white crystals; yield, 92%; mp 170-172 °C; ^{1}H NMR (DMSO-d_6): δ 11.74 (s, 1H, N*H*), 8.36 (s, 1H, H-6), 6.09 (dd, *J* = 8.4, 6.0 Hz, 1H, H-1'), 5.59 (d, *J* = 5.7 Hz, 1H, H-3'), 5.40 (t, *J* = 5.1 Hz, 1H, 5'-O*H*), 4.23 (br d, *J* = 1.8 Hz, 1H, H-4'), 3.62-3.80 (m, 2H, H-5'), 2.40-2.56 (m, 2H, H-2'); ^{13}C NMR (DMSO-d_6): δ 160.13, 149.87, 144.51, 84.34, 84.18, 82.69, 69.76, 61.10, 35.54. Anal. calcd. for $C_9H_{10}IN_3O_7$: C, 27.09; H, 2.53; N, 10.53. Found: C, 27.21; H, 2.68; N, 10.35.

General Method for the Preparation of 5-Substituted-4-(1,2,4-triazolo)-4-deoxy-3'-*O*-nitro-5'-*O*-benzoyl-2'-deoxyuridine (36a-b)

4-Chlorophenyl dichlorophosphate (0.88 mL, 5.32 mmol), and then 1,2,4-triazole (0.74 g, 10.64 mmol), was added to a solution of either **34a** or **34b** (5.32 mmol) in pyridine (20 mL) at 0 °C with stirring. The reaction was allowed to proceed at 25 °C for 4 days with stirring, and the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 (200 mL), this solution was washed with water (2 x 50 mL) and aqueous $NaHCO_3$ (50 mL), the organic fraction was dried (Na_2SO_4), and the solvent was removed in vacuo. Purification of the residue by flash silica gel column chromatography using hexane-EtOAc (1:3 and 1:5, v / v, respectively) gave the respective product **36a** or **36b**. Physical and spectral data for **36a-b** are listed below.

4-(1,2,4-Triazolo)-4-deoxy-3'-*O*-nitro-5'-*O*-benzoyl-2'-deoxyuridine (36a)

Directly as white crystals that were washed with cold ether; yield, 79%; mp 85-87 °C; ^{1}H NMR ($CDCl_3$): δ 9.20 (s, 1H, triazole hydrogen), 8.23 (d, *J* = 7.2 Hz, 1H, H-6),

8.09 (s, 1H, triazole hydrogen), 7.89 (dd, J = 8.7, 1.2 Hz, 2H, *ortho*-benzoyl hydrogens), 7.49-7.58 (m, 1H, *para*-benzoyl hydrogen), 7.32-7.42 (m, 2H, *meta*-benzoyl hydrogens), 6.94 (d, J = 7.2 Hz, 1H, H-5), 6.20 (dd, J = 7.5, 5.7 Hz, 1H, H-1'), 5.65 (d, J = 6.9 Hz, 1H, H-3'), 4.81 (dd, J = 12.0, 3.3 Hz, 1H, H-5'a), 4.72 (dd, J = 5.1, 3.0 Hz, 1H, H-4'), 4.66 (dd, J = 12.0, 3.6 Hz, 1H, H-5'b), 3.18 (ddd, J = 15.3, 7.2, 1.5 Hz, 1H, H-2'α), 2.33-2.43 (m, 1H, H-2'β); ^{13}C NMR ($CDCl_3$): δ 165.72 (CO_2), 159.42 (C-2 C=O), 153.88 (C-4), 145.19 (C-6), 143.18 (phenyl C-1), 133.78, 129.34, 128.77 and 128.68 (phenyl and triazole CH), 94.74 (C-5), 88.47 (C-1'), 82.78 (C-3'), 82.46 (C-4'), 63.85 (C-5'), 38.01 (C-2'). Anal. calcd. for $C_{18}H_{16}N_6O_7$: C, 50.47; H, 3.76; N, 19.62. Found: C, 50.53; H, 3.59; N, 19.71.

4-(1,2,4-Triazolo)-4-deoxy-3'-O-nitro-5'-O-benzoyl-2'-deoxythymidine (36b)

White foam; yield, 71%; mp 59-60 °C; ^{1}H NMR (DMSO-d_6): δ 9.29 (s, 1H, triazole hydrogen), 8.35 (s, 1H, triazole hydrogen), 8.24 (s, 1H, H-6), 7.89 (dd, J = 8.4, 1.5 Hz, 2H, *ortho*-benzoyl hydrogens), 7.57-7.62 (m, 1H, *para*-benzoyl hydrogen), 7.43-7.48 (m, 2H, *meta*-benzoyl hydrogens), 6.15 (dd, J = 7.2, 6.6 Hz, 1H, H-1'), 5.85 (dd, J = 4.8, 2.1 Hz, 1H, H-3'), 4.83 (br dd, J = 6.3, 4.2 Hz, 1H, H-4'), 4.73 (dd, J = 12.3, 3.9 Hz, 1H, H-5'a), 4.60 (dd, J = 12.3, 4.5 Hz, 1H, H-5'b), 2.91 (ddd, J = 15.3, 6.6, 1.5 Hz, 1H, H-2'α), 2.68-2.80 (m, 1H, H-2'β), 2.10 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 165.24, 157.85, 152.71, 147.35, 147.29, 133.39, 129.08, 128.96, 128.92, 128.58, 104.66, 87.76, 83.64, 80.95, 64.14, 36.32, 15.76. Anal. calcd. for $C_{19}H_{18}N_6O_7$: C, 51.59; H, 4.10; N, 19.00. Found: C, 51.45; H, 4.23; N, 19.18.

General Method for the Preparation of 5-Substituted-3'-*O*-nitro-5'-*O*-benzoyl-2'-deoxycytidines (37a-b)

A solution of either **36a**, or **36b** (3.74 mmol) in NH_4OH/dioxane (1:3, v/v, 80 mL) was stirred at 25 °C for 5 h prior to removal of the solvent in vacuo. The residue was purified via flash silica gel column chromatography using MeOH:CH_2Cl_2 (1:9, v/v) as eluent to give the respective products **37a** and **37b**. Physical and spectral data for **37a-b** are listed below.

3'-*O*-Nitro-5'-*O*-benzoyl-2'-deoxycytidine (37a)

Directly as white crystals, which were washed with cold ether; yield, 49%; mp 139-141 °C; ^{1}H NMR (DMSO-d_6): δ 7.98 (dd, *J* = 8.4, 1.2 Hz, 2H, *ortho*-benzoyl hydrogens), 7.68 (ddt, *J* = 14.7, 7.5, 1.2 Hz, 1H, *para*-benzoyl hydrogen), 7.62 (d, *J* = 7.8 Hz, 1H, H-6), 7.51-7.56 (m, 2H, *meta*-benzoyl hydrogens), 7.23 (s, 2H, N*H*$_2$), 6.13 (dd, *J* = 7.8, 6.0 Hz, 1H, H-1'), 5.77 (d, *J* = 4.8 Hz, 1H, H-3'), 5.68 (d, *J* = 7.8 Hz, 1H, H-5), 4.51-4.60 (m, 3H, H-4', H-5'), 2.52-2.61 (m, 2H, H-2'); ^{13}C NMR (DMSO-d_6): δ 165.80, 165.43, 165.24 and 154.57 (quaternary carbons), 140.68 (C-6), 133.34, 129.05, and 128.60 (phenyl *C*H), 94.30 (C-5), 85.60 (C-1'), 83.77 (C-3'), 79.21 (C-4'), 64.19 (C-5'), 35.04 (C-2'). Anal. calcd. for $C_{16}H_{16}N_4O_7$: C, 51.07; H, 4.29; N, 14.89. Found: C, 51.26; H, 4.18; N, 14.75.

5-Methyl-3'-*O*-nitro-5'-*O*-benzoyl-2'-deoxycytidine (37b)

Directly as white crystals, which were washed with cold ether; yield, 36%; mp 158-160 °C; ^{1}H NMR (DMSO-d_6): δ 8.15 (d, *J* = 8.1 Hz, 2H, *ortho*-benzoyl hydrogens),

7.76-7.85 (m, 1H, *para*-benzoyl hydrogen), 7.59-7.71 (m, 2H, *meta*-benzoyl hydrogens), 7.55 (br s, 1H, N*H*a), 7.49 (s, 1H, H-6), 7.00 (br s, 1H, N*H*b), 6.32 (dd, J = 8.1, 6.0 Hz, 1H, H-1'), 5.90-5.96 (m, 1H, H-3'), 4.63-4.79 (m, 3H, H-4', H-5'), 2.60-2.70 (m, 2H, H-2'), 1.77 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 165.30, 165.22, 165.16, 154.66, 137.54, 133.48, 129.06, 128.73, 101.81, 84.98, 83.87, 79.08, 64.22, 35.04, 12.90. Anal. calcd. for $C_{17}H_{18}N_4O_7$: C, 52.31; H, 4.65; N, 14.35. Found: C, 52.06; H, 4.41; N, 14.57.

General Method for the Preparation of 5-Substituted-3'-*O*-nitro-2'-deoxycytidines (38a-b)

A solution of NaOMe in MeOH (3.7 mL of 0.5 M) was added to either **37a**, or **37b** (0.93 mmol), the mixture was stirred at 25 °C for 15 min, and the solvent was removed in vacuo. Purification of the residue by flash silica gel column chromatography using MeOH:CH_2Cl_2 (1:4, v/v) as eluent afforded the respective products **38a** and **38b**. Physical and spectral data for **38a-b** are listed below.

3'-*O*-Nitro-2'-deoxycytidine (38a)

White foam; yield, 95%; mp 65-66 °C [Lit (Huang *et al.*, 1978) mp 177-178 °C]; ^{1}H NMR (DMSO-d_6): δ 7.80 (d, J = 7.8 Hz, 1H, H-6), 7.20 and 7.26 (two s, 1H each, NH_2), 6.14 (dd, J = 8.7, 5.4 Hz, 1H, H-1'), 5.76 (d, J = 7.8 Hz, 1H, H-5), 5.59 (d, J = 6.0 Hz, 1H, H-3'), 5.25 (t, J = 5.4 Hz, 1H, 5'-O*H*), 4.19 (br d, J = 1.8 Hz, 1H, H-4'), 3.62-3.66 (m, 2H, H-5'), 2.45-2.52 (m, 1H, H-2'α), 2.29 (ddd, J = 14.7, 6.0, 2.7 Hz, 1H, H-2'β); ^{13}C NMR (DMSO-d_6): δ 163.67, 152.34, 141.70, 94.37, 85.02, 84.80, 82.78, 61.09,

35.88. Anal. calcd. for $C_9H_{12}N_4O_6$: C, 39.71; H, 4.44; N, 20.58. Found: C, 39.75; H, 4.38; N, 20.60.

5-Methyl-3'-*O*-nitro-2'-deoxycytidine (38b)

Colorless oil, which was crystallized from EtOAc-hexane to give white crystals; yield, 94%; mp 141-143 °C; ^{1}H NMR (DMSO-d_6): δ 7.62 (s, 1H, H-6), 7.35 and 6.90 (two s, 1H each, N*H*$_2$), 6.14 (dd, *J* = 9.0, 5.7 Hz, 1H, H-1'), 5.57 (d, *J* = 5.1 Hz, 1H, H-3'), 5.35 (br s, 1H, 5'-O*H*), 4.16 (d, *J* = 2.1 Hz, 1H, H-4'), 3.60-3.70 (m, 2H, H-5'), 2.41-2.49 (m, 1H, H-2'α), 2.25-2.36 (m, 1H, H-2'β), 1.84 (s, 3H, C*H*$_3$); ^{13}C NMR (DMSO-d_6): δ 165.40 (C-2 *C*=O), 155.10 (C-4), 138.12 (C-6), 101.96 (C-5), 84.88 and 84.79 (C-1' and C-3'), 82.36 (C-4'), 61.45 (C-5'), 35.70 (C-2'), 13.30 (*C*H$_3$). Anal. calcd. for $C_{10}H_{14}N_4O_6$: C, 41.96; H, 4.93; N, 19.57. Found: C, 42.29; H, 4.68; N, 19.86.

5-Iodo-3'-*O*-nitro-2'-deoxycytidine (38c)

A mixture of **38a** (0.144 g, 0.53 mmol), iodine (0.135 g, 0.53 mmol) and iodic acid (0.093 g, 0.53 mmol) in acetic acid, H_2O and CCl_4 (8:3:2, v/v/v, 26 mL) was stirred at 25 °C for 14 h. Removal of the solvents in vacuo, co-evaporation of toluene (2 x 20 mL) from the residue, dissolution of the residue in CH_2Cl_2 (100 mL), filtration, washing with aqueous $NaHCO_3$ (50 mL), and drying the organic fraction (Na_2SO_4) were carried out consecutively. The solvent was removed in vacuo to give a residue that was purified via flash silica gel column chromatography using MeOH:CH_2Cl_2 (1:9, v/v) as eluent to afford **38c** (0.118 g, 56%) as a white foam: mp 81-82 °C; ^{1}H NMR (DMSO-d_6): δ 8.23 (s, 1H, H-6), 7.95 and 6.75 (two br s, 1H each, N*H*$_2$), 6.09 (dd, *J* = 8.7, 5.7 Hz, 1H, H-1'),

5.59 (d, J = 6.0 Hz, 1H, H-3'), 5.36 (t, J = 5.4 Hz, 1H, 5'-O*H*), 4.20-4.28 (m, 1H, H-4'), 3.62-3.76 (m, 2H, H-5'), 2.49-2.56 (m, 1H, H-2'α), 2.36 (ddd, J = 15.0, 8.4, 6.6 Hz, 1H, H-2'β); ^{13}C NMR (DMSO-d_6): δ 163.58, 153.61, 147.07, 85.11, 84.83, 82.63, 61.13, 57.04 (C-5), 36.10. Anal. calcd. for $C_9H_{11}IN_4O_6$: C, 27.15; H, 2.78; N, 14.07. Found: C, 27.22; H, 2.70; N, 14.16.

General Method for the Preparation of 5-Substituted-5'-*O*-nitro-2'-deoxyuridines (40a-b)

A mixture of either **39a** or **39b** (6.54 mmol) and $LiNO_3$ (4.48 g, 65 mmol) in dry DMF (30 mL) was stirred at 100 °C for 14 hours under argon. Removal of the solvent in vacuo gave a residue, toluene (20 mL) was added, and the solvent was removed in vacuo. Purification of the residue via flash silica gel column chromatography using a gradient of 5% →10% MeOH in CH_2Cl_2 as eluent gave a pale yellow oil which was recrystallized from MeOH to yield the respective product **40a** or **40b**. Physical and spectral data for **40a-b** are listed below.

5'-*O*-Nitro-2'-deoxyuridine (40a)

Yield, 73%; mp 170-172 °C [Lit (Lichtenthaler *et al.*, 1973) mp 175 °C]; ^{1}H NMR (DMSO-d_6): δ 11.34 (s, 1H, N*H*), 7.65 (d, J = 8.1 Hz, 1H, H-6), 6.16 (dd, J = 7.2, 6.6 Hz, 1H, H-1'), 5.65 (d, J = 8.1 Hz, 1H, H-5), 5.53 (d, J = 3.9 Hz, 1H, 3'-O*H*), 4.79 (dd, J = 11.4, 3.6 Hz, 1H, H-5'a), 4.66 (dd, J = 11.4, 6.9 Hz, 1H, H-5'b), 4.20-4.27 (m, 1H, H-3'), 3.95-4.00 (m, 1H, H-4'), 2.09-2.28 (m, 2H, H-2'); ^{13}C NMR (DMSO-d_6) δ

162.81, 150.19, 140.65, 101.98, 84.59, 81.76, 73.05 (C-5'), 70.10, 38.19. Anal. calcd. for $C_9H_{11}N_3O_7$: C, 39.57; H, 4.06; N, 15.38. Found: C, 39.46; H, 3.91; N, 15.57.

5'-*O*-Nitro-2'-deoxythymidine (40b)

Yield, 71%; mp 183-185 °C [Lit (Schwandt *et al.*, 1968), mp 189 °C]; ^{1}H NMR (DMSO-d_6): δ 11.32 (s, 1H, N*H*), 7.46 (s, 1H, H-6), 6.19 (t, *J* = 6.9 Hz, 1H, H-1'), 5.51 (d, *J* = 3.3 Hz, 1H, 3'-O*H*), 4.81 (dd, *J* = 11.4, 3.9 Hz, 1H, H-5'a), 4.69 (dd, *J* = 11.4, 6.6 Hz, 1H, H-5'b), 4.28-4.30 (m, 1H, H-3'), 3.96 (ddd, *J* = 6.6, 3.9, 3.6 Hz, 1H, H-4'), 2.25 (ddd, *J* = 14.1, 7.2, 6.9 Hz, 1H, H-2'α), 2.11 (ddd, *J* = 14.1, 6.3, 3.9 Hz, 1H, H-2'β), 1.78 (s, 3H, C*H*$_3$); ^{13}C NMR (DMSO-d_6): δ 163.50, 150.28, 135.99, 109.79, 84.08, 81.69, 73.07 (C-5'), 70.26, 38.08, 12.07. Anal. calcd. for $C_{10}H_{13}N_3O_7$: C, 41.82; H, 4.56; N, 14.63. Found: C, 41.96; H, 4.68; N, 14.74.

5-Iodo-5'-*O*-nitro-2'-deoxyuridine (40c)

A mixture of **40a** (0.2 g, 0.74 mmol) and NaN_3 (0.19 g, 2.94 mmol) in dry MeCN (20 mL) was cooled in an ice-bath. A solution of ICl (0.36 g, 2.2 mmol) in MeCN (3 mL) was added drop wise during 5 min, and the reaction was allowed to proceed at 25 °C for 48 h with stirring under argon. The solvent was removed in vacuo and the residue was purified via silica gel flash column chromatography using a gradient of 5% → 10% MeOH in CH_2Cl_2 as eluent to give **40c** (0.19 g, 65%) as white crystals: mp 181-183°C; ^{1}H NMR (DMSO-d_6): δ 11.80 (s, 1H, N*H*), 8.10 (s, 1H, H-6), 6.18 (dd, *J* = 6.9, 3.9 Hz, 1H, H-1'), 5.59 (d, *J* = 4.2 Hz, 1H, 3'-O*H*), 4.75-4.94 (m, 2H, H-5'), 4.33-4.37 (m, 1H, H-3'), 4.06 (ddd, *J* = 6.9, 6.6, 3.6 Hz, 1H, H-4'), 2.40 (ddd, *J* = 14.1, 7.2, 6.9 Hz, 1H, H-

2'α), 2.19 (ddd, J = 14.1, 6.6, 3.9 Hz, 1H, H-2'β); ^{13}C NMR (DMSO-d_6): δ 160.21(C-4 C=O), 149.86 (C-2 *C*=O), 144.80 (C-6), 85.18 (C-1'), 82.08 (C-3'), 72.85 (C-5'), 70.13 (C-4'), 69.78 (C-5), 38.32. Anal. calcd. for $C_9H_{10}IN_3O_7$: C, 27.09; H, 2.53; N, 10.53. Found: C, 29.27; H, 2.49; N, 10.58.

1-(2'-Deoxy-β-D-ribofuranosyl)-5-(2-bromo-1-hydroxyethyl)-2,4-difluorobenzene (45)

N-Bromosuccinimide (0.037 g, 0.211 mmol) was added slowly with stirring to a solution of **44** (0.052 g, 0.205 mmol) in dioxane-water (3:7, v/v, 3 mL) and glacial acetic acid (20 μL) during a period of 5 min, and the reaction was allowed to proceed at 25°C for 2 h with stirring. Removal of the solvent in vacuo gave a residue that was purified by flash silica gel column chromatography using hexane-EtOAc (1:2, v/v) as eluent to yield **45** as a white solid (0.054 g, 76%) after recrystallization from ether-hexane; mp 97-99°C; ^{1}H NMR ($CDCl_3$) (mixture of two diastereomers in a ratio of 1:1): δ 7.65 (dd, J = 8.2, 7.6 Hz, 1H , H-6), 6.80 (t, J = 10.2 Hz, 1H, H-3), 5.35 (dd, J =10.2, 6.2 Hz, 1H, H-1'), 5.20 (dd, J = 8.5, 3.0 Hz, 1H, C*H*OHCH$_2$Br), 4.42-4.52 (m, 1H, H-3'), 4.00-4.08 (m, 1H, H-4'), 3.82-3.88 (complex m, 1H, C*H*H'Br), 3.74-3.78 (complex m, 1H, CH*H'*Br), 3.67 and 3.69 (two dd, J =10.0, 3.5 Hz, 1H total, H-5'a), 3.49 and 3.51 (two dd, J =10.0, 1.5 Hz, 1H total, H-5'b), 2.28-2.40 (m, 1H, H-2'α), 1.98-2.12 (m, 1H, H-2'β), 1.83 (br s, 3H, O*H*); ^{13}C NMR (DMSO-d_6): δ 159.26 (dd, J = 248.3, 13.2 Hz, C-2), 156.57 (dd, J = 247.2, 12.1 Hz, C-4), 125.21-125.49 (multiple lines, C-6), 125.21-125.49 (multiple lines, C-1 and C-5), 102.99 (dd, J = 26.4, 25.3 Hz, C-3), 87.46 (C-1'), 72.62 (C-3'), 72.06 (C-

4'), 65.89 and 66.08 (*C*HOH), 62.17 (C-5'), 41.83 and 41.90 (C-2'), 38.00 and 38.07 (*C*H_2Br). Anal. calcd. for $C_{13}H_{15}BrF_2O_4$: C, 44.19; H, 4.25. Found: C, 44.14; H 4.03.

1-(2'-Deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-(1-hydroxy-2-iodoethyl)benzene (46)

A solution of **44** (0.054 g, 0.21 mmol), iodine (0.026 g, 0.2 mmol), potassium iodate (0.011 g, 0.05 mmol), acetonitrile (1 mL) and sulfuric acid (50 μL of 5N) was maintained at 60°C for 3 h with stirring. Removal of the solvent in vacuo gave a residue which was purified by flash silica gel column chromatography using hexane-EtOAc (1:2, v/v) as eluent to yield **46** (0.060 g, 71%) as a white solid, after recrystallization from ether-hexane; mp 103-105°C; ^{1}H NMR ($CDCl_3$) (mixture of two diastereomers in a ratio of 1:1): δ 7.58-7.68 (m, 1H, H-6), 6.75 (t, *J* =10.0 Hz, 1H, H-3), 5.32 (dd, *J* = 10.1, 6.1 Hz, 1H, H-1'), 5.04 and 5.06 (two dd, J = 6.6, 3.7 Hz, 1H total, C*H*OHCH_2I), 4.43-4.50 (m, 1H, H-3'), 3.98-4.06 (m, 1H, H-4'), 3.87 (ddd , *J* =11.5, 4.0, 2.1 Hz, 1H, H-5'a), 3.75 (dd, *J* =11.5, 5.4 Hz, 1H, H-5'b), 3.50-3.60 (complex m, 1H, C*H*H'I), 3.32-3.42 (complex m, 1H, CH*H'*I), 2.26-2.40 (m, 1H, H-2'α), 1.98-2.12 (m, 1H, H-2'β), 1.79 (br s, 3H, O*H*), ^{13}C NMR (DMSO-d_6 + $CDCl_3$): δ 159.42 (*J* = 248.3, 13.2 Hz, C-2), 156.60 (*J* = 247.2, 12.1 Hz, C-4), 125.75-125.97 (multiple lines, C-6), 124.98-125.57 (multiple lines, C-1 and C-5), 102.36 (dd, *J* = 27.5, 26.4 Hz, C-3), 87.21 (C-1'), 72.63 and 72.75 (C-3'), 72.08 and 72.11 (C-4'), 65.97 and 66.39 (*C*HOH), 62.28 (C-5'), 42.05 (C-2'), 12.58 and 13.06 (*C*H_2I). Anal. calcd. for $C_{13}H_{15}F_2IO_4$: C, 39.02; H, 3.78. Found: C, 39.12; H 4.10.

1-(2'-Deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-(2-iodo-1-methoxyethyl)benzene (47)

A solution of iodine monochloride (0.051 g, 0.312 mmol) in methanol (1 mL) was added to a solution of **44** (0.040 g, 0.156 mmol) in methanol (2 mL) with stirring, and the reaction mixture was heated at 50°C for 2 h. Removal of the solvent in vacuo gave a residue which was purified by flash silica gel column chromatography using hexane-EtOAc (1:1 to 1:2, v/v) as eluent to give a yellow oil (0.042 g) which was recrystallized from ether-hexane to afford **47** (0.036 g, 56%) as white crystals; mp 96-98°C; ^{1}H NMR ($CDCl_3$) (mixture of two diastereomers in a ratio of 1:1): δ 7.40-7.46 (m, 1H, H-6), 6.70 (t , J = 10.2 Hz, 1H , H-3), 5.27 (dd, J = 10.1, 6.1 Hz, 1H, H-1'), 4.48-4.52 (m, 1H, H-3'), 4.40-4.43 (m, 1H, C*H*OMe), 3.94-3.98 (m, 1H, H-4'), 3.79 (dd, J =11.5, 3.5 Hz, 1H, H-5'a), 3.70 (dd, J = 11.5, 4.5 Hz, 1H, H-5'b), 3.21-3.42 (complex m, 2H, CH_2I), 3.26 and 3.27 (two s, 3H total, OCH_3), 2.22-2.32 (m, 1H, H-2'α), 1.92-2.04 (m, 1H, H-2'β); ^{13}C NMR ($CDCl_3$): δ 78.43 (*C*HOMe), 57.36 (O*C*H$_3$), 9.53 (*C*H$_2$I). Anal. calcd. for $C_{14}H_{17}F_2IO_4.H_2O$: C, 38.88; H, 4.39. Found: C, 38.53; H 4.01.

1-(2'-Deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-oxiranylbenzene (48)

Method A: A solution of **45** (0.064 g, 0.182 mmol) in dioxane-water (3:7, v/v, 3 ml) and NaOH (0.01 g, 0.25 mmol) was stirred at 25°C for 45 min, the solvent was removed in vacuo, and the residue was extracted with EtOAc (30 mL). Removal of the solvent in vacuo afforded a residue, which was purified by flash silica gel column chromatography using hexane-EtOAc (1:3, v/v) as eluent to afford **48** (0.035 g, 71%) as a white solid, after recrystallization from ether-hexane; mp 83-85°C; ^{1}H NMR ($CDCl_3$): δ 7.18-7.30 (m, 1H, H-6), 6.78 (t, J = 10.1 Hz, 1H, H-3), 5.27 (dd, J = 9.8, 4.9 Hz, 1H, H-

1'), 4.36-4.48 (m, 1H, H-3'), 4.04-4.11 (m, 1H, H-4'), 3.99 (dd, *J*gem = 5.2, *J*vic = 2.5 Hz, 1H, oxiranyl C*H*), 3.62-3.80 (m, 2H, H-5'a and H-5'b), 3.13 (dd, *J*gem = 5.5, *J* vic = 5.2 Hz, 1H, oxiranyl C*H*H'), 2.76 and 2.79 (two dd, *J*gem = 5.5, *J*vic = 2.5 Hz, 1H, oxiranyl CH*H'*), 2.20-2.34 (m, 1H, H-2'α), 1.90-2.08 (m, 1H, H-2'β); ^{13}C NMR ($CDCl_3$): δ 162.34 (dd, *J* = 262.5, 13.8 Hz, C-2), 159.17 (dd, *J* = 262.5, 13.8 Hz, C-4), 125.26 and 125.07 (C-6), 120.78 and 120.99 (C-5 and C-1), 104.15 and 103.81 (two dd, *J* = 26.4, 25.3 Hz, C-3), 88.15 (C-4'), 74.49 and 74.35 (C-1'), 74.05 (C-3'), 63.21 (C-5'), 50.28 and 50.15 (oxiranyl *C*H_2), 47.05 and 46.97 (oxiranyl *C*H), 42.69 and 42.56 (C-2'). Anal. calcd. for $C_{13}H_{14}F_2O_4.1/3H_2O$: C, 56.11; H, 5.27. Found: C, 56.07; H, 5.06.

Method B: A solution of **46** (0.040 g, 0.1 mmol) in dioxane-water (3:7, v/v, 2 mL) and NaOH (0.006 g, 0.15 mmol) was stirred at 25°C for 30 min. The solvent was removed in vacuo and the residue was purified by flash silica gel column chromatography using hexane-EtOAc (1:3, v/v) as eluent to yield **48** (0.022 g, 81%) as white crystals, which was identical (mp, ^{1}H NMR) to **48** described above under Method A.

1-(2'-Deoxy-β-D-ribofuranosyl)-5-(2,2-dibromo-1-hydroxyethyl)-2,4-difluorobenzene (51)

N-Bromosuccinimide (0.023 g, 0.130 mmol) was added slowly with stirring to a solution of **49** (0.040 g, 0.119 mmol) in dioxane-water (3:7, v/v, 2 mL) and glacial acetic acid (20 μL) during a period of 5 min, and the reaction was allowed to proceed at 25°C for 4 h with stirring. Removal of the solvent in vacuo gave a residue that was purified by flash silica gel column chromatography using hexane-EtOAc (1:2, v/v) as eluent to give a

pale yellow oil (0.050 g), which was recrystallized from ether-hexane to afford **51** (0.044 g, 85%) as white crystals; mp 60-62°C; ^{1}H NMR (CD_3OD) (mixture of two diastereomers in a ratio of 1:1): δ 7.78-7.90 (m, 1H, H-6), 6.92 (t, J = 10.4 Hz, 1H, H-3), 6.02 and 6.05 (two d, J = 5.2 and 3.9 Hz, 1H total, C*H*Br_2), 5.25-5.38 (m, 1H, H-1'), 5.18 and 5.20 (two d, J = 5.2 and 3.9 Hz, 1H total, C*H*OHCHBr_2), 4.28-4.34 (m, 1H, H-3'), 3.88-3.95 (m, 1H, H-4'), 3.62-3.73 (m, 2H, H-5'), 2.20-2.30 (m, 1H, H-2'α), 1.82-1.96 (m, 1H, H-2'β); ^{13}C NMR (CD_3OD): δ 162.2 (dd , J = 252.6, 13.2 Hz, C-2) ; 159.34 (dd, J = 252.6, 12.9 Hz, C-4), 128.62 and 128.53 (C-6), 126.92 (C-5), 125.19 (C-1), 104.36 and 104.02 (two t, J = 26.4 Hz, C-3), 88.95 (C-4'), 75.00 and 74.78 (C-1'), 74.21 and 74.17 (C-3'), 73.46 and 73.21 (*C*HOHCHBr_2), 63.95 (C-5'), 51.12 and 50.86 (*C*HBr_2), 43.61 and 43.48 (C-2'). Anal. calcd. for $C_{13}H_{14}Br_2F_2O_4$: C, 36.11; H, 3.24. Found: C, 36.35; H 3.46.

1-(2'-Deoxy-β-D-ribofuranosyl)-2,4-difluoro-5-(2,2-diiodo-1-hydroxyethyl)benzene (52)

A solution of **50** (0.065 g, 0.172 mmol), iodine (0.022 g, 0.17 mmol), potassium iodate (0.01 g, 0.047 mmol), acetonitrile (3 mL), water (2 mL) and sulfuric acid (15 μL of 5N) was maintained at 60°C for 4 h with stirring. Removal of the solvent in vacuo gave a residue which was purified by flash silica gel column chromatography using hexane-EtOAc (1:2, v/v) as eluent to give a yellow oil (0.085 g), which was recrystallized from ether-hexane to yield **52** (0.081 g, 90%) as white crystals; mp 68-70°C; ^{1}H NMR (CD_3OD) (mixture of two diastereomers in a ratio of 1:1): δ 7.72-7.84 (m, 1H, H-6), 6.89 (t, J = 10.2 Hz, 1H, H-3), 5.42-5.50 (m, 1H, C*H*I_2), 5.32 (dd, J = 10.4, 5.7 Hz, 1H, H-1'),

4.86-4.96 (m, 1H, C*H*OHCHI$_2$), 4.24-4.32 (m, 1H, H-3'), 3.88-3.98 (m, 1H, H-4') ; 3.60-3.72 (m, 2H, H- 5'), 2.18-2.32 (m, 1H, H-2'α), 1.82-1.96 (m, 1H, H-2'β): ^{13}C NMR (CD_3OD): δ 164.47 (dd, J = 251.3, 12.6 Hz, C-2), 159.04 (dd, J = 252.1, 13.3 Hz, C-4), 128.34 and 128.21 (C-6), 126.77 and 126.60 (C-5), 125.74 (C-1), 104.41 (t, J = 26.4 Hz, C-3), 88.93 (C-4'), 75.06 (C-1'), 74.40 and 74.24 (C-3'), 74.02 (*C*HOHCHI$_2$), 63.98 (C-5'), 43.58 and 43.15 (C-2'), −15.77 (*C*HI$_2$). Anal. calcd. for $C_{13}H_{14}F_2I_2O_4$: C, 29.66; H, 2.66. Found: C, 29.85; H 2.80.

In vitro cell cytotoxicity (MTT assay)

KBALB, KBALB-STK, human 143B, human 143B-LTK and R-970-5 cells were cultured in complete DMEM medium supplemented with 10% fetal bovine serum (FBS), and EMT-6 cells were cultured in complete WAYMOUTH medium in 10% FBS. Exponentially growing cells were trypsinized, centrifuged, resuspended in growth medium, and the cell number was readjusted to 8 x 10^3 cells/mL. Cells were seeded into 96-well plates at 8 x 10^2 cells/well, and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 h.

The test compound was dissolved in DMEM medium, and 100 μL of this solution was added to cells in 96-well plates to produce the preselected test compound concentration. DMEM medium (100 μL) was added to control wells. The plates were incubated for 3 days at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. At the end of the incubation, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma) was dissolved in phosphate-buffered saline (PBS) to produce a concentration of 5 mg/mL, filtered through a 0.45 μm membrane filter, and

diluted (1:5) with pre-warmed DMEM medium. A 50 μL aliquot of this solution was added to each well, and the plates were incubated at 37 °C for 4 h. The medium was removed from the wells, dimethyl sulfoxide (150 μL) was added to each well, and the plates were placed on a shaker for 15 min to dissolve the formazan crystals. The absorbance at 540 nm (A_{540}) was measured immediately in each well using a scanning multi-well spectrophotometer (ELISA reader). A_{540} values, corrected for the absorbance in medium blanks, reflected the concentration of viable cells. The CC_{50} values reported are the test drug concentration that reduced the A_{540} to 50% of the control value (mean value, n = 6). This assay (Alley *et al.*, 1988), which depends on the metabolic reduction of MTT to colored formazan, measures cytostatic and cytotoxic effects of the test drug.

Antiviral activity assays

The antiviral assays were based on an inhibition of virus-induced cytopathicity in either E_6SM, HeLa or Vero cell cultures, following previously established procedures (De Clercq, 1994). Herpes simplex virus type 1 (HSV-1) (KOS), HSV-2 (G), vaccinia virus, vesicular stomatitis virus (VSV), and the thymidine kinase-deficient HSV-1 TK^- KOS (ACV^r) strains were propagated in E_6SM cell cultures, parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie B4 virus and Punta Toro virus in Vero cell cultures, and respiratory syncytial virus in HeLa cell cultures. Briefly, confluent cell cultures in microtiter plates were exposed to 100 $CCID_{50}$ of virus, 1 $CCID_{50}$ being the virus dose required to infect 50% of the cell cultures. After a 1 h virus adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying

concentrations (400, 100, .. μg/mL) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures.

Inhibition of HIV-induced giant cell formation

CEM cell cultures were suspended at 250,000-300,000 cells/mL of culture medium, and infected with HIV-1(III_B) or HIV-2(ROD) at 100 $CCID_{50}$/mL. Then, 100 μL of the infected cell suspension were transferred to 200 μl microtiter plate wells containing 100 μL of serial dilutions of the test compound solutions. After 4 days of incubation at 37°C, cell cultures were examined for syncytium formation as previously described (Balzarini *et al.*, 1991).

In vitro nitric oxide release assays

1. Incubation with 18 mM L-cysteine in phosphate buffer (pH 7.4)

In vitro nitric oxide release was assayed using a modification of the previously reported procedure (Sako *et al.*, 1998). Briefly, a solution of the test compound (1 mL of a 2 mM solution in 0.1 M phosphate buffer (pH 7.4) was mixed thoroughly with a freshly prepared solution of L-cysteine (1 mL of a 36 mM solution in 0.1 M phosphate buffer, pH 7.4), and the mixture was incubated at 37 ºC, for 1 and 16 h in the absence of air. After exposure to air for 10 min at 25 °C, an aliquot of the Griess reagent (1 mL) [freshly prepared by mixing equal volumes of 1.0% sulfanilamide (prepared and stored in aqueous 5% phosphoric acid) and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in water] was added to an equal volume (1 mL) of each test compound's incubation solution with mixing. After 10 min had elapsed, absorbance was measured at 540 nm

using a Philips PU 8740 UV/VIS scanning spectrophotometer. Solutions of 0-60 μM sodium nitrite were used to prepare a nitrite absorbance versus concentration curve under the same experimental conditions. The percent nitric oxide released (quantitated as nitrite ion) was calculated (± SEM, n = 3) from the standard nitrite versus concentration curve.

2. Incubation with phosphate buffer (pH 7.4)

This assay was performed as described under procedure 1 above, except that a solution of the test compound (2 mL of a 2 mM solution in 0.1 M phosphate buffer pH 7.4) was used, and no L-cysteine was added.

3. Incubation with rat serum

Nitric oxide release was measured by modification of the reported procedure for in vivo serum samples (Grisham & Johnson, 1996). Briefly, rat serum was prepared by filtration with a Millipore UL trafree-15 centrifugal filter. Stock solutions of test compounds (1×10^{-2} M) were prepared, and 10 μL of each test compound solution was mixed with filtered serum (90 μL), which corresponds to the reaction sample. Each reaction sample was incubated for either 1h, or 16h, at 37 °C in the presence of 0.2 U/mL Aspergillus nitrate reductase, 50 mM HEPES buffer, 5 μM FAD and 0.1 mM NADPH to provide a total volume of 500 μL that was comprised of the following composition for the blank and the reaction sample (in brackets), respectively: water 300 μL (290 μL), 1 M HEPES 25 μL (25 μL), serum 90 μL (100 μL), 0.1 mM FAD 25 μL (25 μL), 1 mM NADPH 50 μL (50 μL), 10U/mL Nase 10 μL (10 μL). Following the incubation, 5 μL of lactate dehydrogenase (1500 U/mL) and 50 μL of 100 mM pyruvic acid were added to

each tube to oxidize any unreacted NADPH, and samples were incubated for 10 min. Griess reagent (550 μL) was then added to each tube, the mixture was allowed to stand at 25 °C for 10 min, absorbance for each sample was determined at 540 nm, and % nitric oxide release was calculated as indicated under Procedure 1 described above.

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